REFERENCE ONLY

UNIVERSITY OF LONDON THESIS

Degree PhD Year 2000 Name of Author O'MAHONY, MONA

NOVEL INHIBITORS OF ADHESIN-RECEPTOR INTERACTIONS INVOLVED IN MICROBIAL INFECTION AT MUCOSAL SURFACES

Rachel Mary O'Mahony

PhD Thesis

MICROBIOLOGY

Department of Infection,
Centre for Infectious Diseases & International Health,
University College London

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ACKNOWLEDGEMENTS

I am indebted to and wish to thank my supervisors Dr. John Holton and Dr. Christelle Basset for their endless and invaluable help, support and encouragement during this project, without whom this would not have been possible. I also wish to thank Prof. Ivan Roitt for his kindness as well as guidance and supervision over the ADRI part of this project.

I wish to thank my family who I am also indebted to for their patience and providing the endless support I have needed throughout my research. To my dear friends Susan Chang, Rena Singh, Shali Vidyadaran and Zeta Anagnostou - together we have shared the 'experience' of doing a PhD. To Fatima Miranda and Helen Donoghue for their endless support in and out of the lab.

Many thanks to Prof. Dino Vaira for providing the gastric tissue used throughout the project and to Prof. Arnoud van Vliet for providing *H. pylori* GP15 mutants for the regions study. Thanks to all the collaborators of the ADRI project, in particular Dr. Justine Younsen, Dr. Haiqun Lui, Prof. Thomas Boren, Dr. Colin Campion and Anna Hultberg for all their help, advice and correspondance throughout the project.

I am also grateful to Huda Al-Khtheeri for assistance with the plants study, to Martin Avedian for the idea of testing borage and for its supply and to Ms. A. Al-Dossary for supplying black caraway. I also wish to thank Mariam Andraweiss and Nalini Singh for technical advice.

I would like to acknowledge the European Union on EC contract QLK2-CT-2001-01216 (ADRI) for financial support.

Finally, I wish to thank God for being my overall source of guidance and strength during this project, without whom I would not have come this far.

ABSTRACT:

Microorganisms are becoming increasingly resistant to current antimicrobial agents and therefore new strategies and agents are being developed to combat infection. One strategy is to target and block the first step of microbial infection (adhesion of the microbe to the host tissue) by using molecules that mimic (or antibodies against) the microbial adhesin or its complementary host cell receptor. Plants have also been shown to provide natural sources of antimicrobial substances as well as inhibit microbial adhesion. One major problem in adhesion-inhibition studies has been the accurate quantification of adhesion. Most investigators have relied on manual counting while a few have used automated methods using image analysis software.

The first aim of this study was therefore to compare the effectiveness of several current software packages, to develop the most accurate method of quantification and to use this method to test potential inhibitors of microbial adhesion. The organisms under investigation in this project were *Candida albicans* and *Helicobacter pylori*, both of which are becoming resistant to available antibiotic treatments.

A new and accurate method of quantification was developed for assessing microbial adhesion using 'Metamorph' image analysis software. Aided by this system, several domain antibodies, carbohydrates and plant extracts were found to be successful inhibitors of *C. albicans* and *H.pylori* adhesion *in vitro* and therefore have the potential to form the basis of new and alternative therapies to treat infection caused by these microorganisms.

Additionally, because it is not fully known why *H. pylori* preferentially colonises specific topographical regions of the human stomach, the second aim was to compare its adhesion to different topographical regions of the stomach, in an attempt to explain this phenomenon. No difference was found between the receptors present in the antrum or fundus of inflamed human stomachs. However, further investigations involving both inflamed and non-inflamed stomachs are warranted.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS AND PUBLICATIONS

List of Figures:

List of Tables:

List of Publications:

Journal articles

Basset C, Holton J, **O'Mahony R** and Roitt I. **2003**. Innate immunity and pathogen-host interaction. *Vaccine* **21** Suppl 2: 512-23.

O'Mahony R, Vaira D, Holton J and Basset C. **2004**. *Helicobacter pylori*: Current status and future prospects. *Sci. Prog.* **87**: 269-96.

O'Mahony R, Basset C, Holton J, Vaira D and Roitt I. **2005**. Comparison of image analysis software packages in the assessment of adhesion of microorganisms to mucosal epithelium using confocal laser scanning microscopy. *J. Microbiol. Methods* **61**: 105-26.

O'Mahony R, Al-Khtheeri, Weerasekera D, Fernando N, Vaira D, Holton J and Basset C. **2005.** Bactericidal and anti-adhesive properties of culinary and medicinal plants against *Helicobacter pylori. World J. Gastroenterol* **11**:7499-507.

DeBernardis F, **O'Mahony R**, Li H, Basset C, Holton J, Roitt I, Cassone A. Human hypervariable antibody domains recognizing virulence traits of *Candida albicans* confer passive protection against experimental vaginal candidiasis. Submitted to *Nature Biotechnology.*

Abstracts and posters:

Posters presented at the European Helicobacter Study Group XVII International workshop, September 2004 in Vienna, Austria. Abstracts of the posters were published in *Helicobacter* **2004**; **9**: 489-90.

O'Mahony R, Basset C, Holton J, Vaira D and Roitt I. Comparison of image analysis software packages in the assessment of adhesion of microorganisms to mucosal epithelium using confocal laser scanning microscopy.

O'Mahony R, Shinokubo S, Weerasekera D, Bogahawata A, Fernando N, Vaira D, Holton J and Basset C. Bactericidal and anti-adhesive properties of culinary and medicinal plants against *Helicobacter pylori.*

Other:

Interview with scientific journalsist for article 'Making software count', published in *Biophotonics International* magazine, April **2005**:17-18.

Publications can be found inside the back cover of the thesis

List of Abbreviations:

ATCC	American Type Culture Collection
Alp	Adherence associated lipoproteins
ALS	Agglutin-like sequence
APC	Antigen-presenting cell
APP	Acute phase protein
AR	Antigen retrieval
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BabA	Blood group antigen binding adhesin
BC	Bovine colostrum
BEC	Buccal epithelial cell
BSA	Bovine serum albumin
CagA	Cytotoxin associated gene A
CAG	Chronic active gastritis
CDC	Centers for Disease Control
CFU	Colony forming units
CG	Chronic gastritis
CLSM	Confocal laser scanning microscopy
CSH	Cell surface hydrophobicity
dAb	Domain antibody
DAB	Diaminobenzidine
DC	Dendritic cell
DU	Duodenal ulcer
EAR	Electrochemical antigen retrieval
EC	Epithelial cell
ECL	Enterochromaffin-like cells
ECM	Extracellular matrix
EPEC	Enteropathogenic *E. coli*
Fab	Fragment antigen-binding
FITC	Fluorescein isothiocyanate
FSC	Forward scatter

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Gastric cancer
GD	Ganglioside
GI	Gastrointestinal
GM	Ganglioside
Gg3	Gangliotriaosylceramide
Gg4	Gangliotetrasylceramide
HBEC	Human buccal epithelial cell
HIER	Heat induced epitope retrieval
HopZ	*H. pylori* outer membrane protein Z
HpaA	*H. pylori* adhesin A
Hp-NAP	*H. pylori* neutrophil activating protein
HRP	Horse radish peroxidase
HSA	Human serum albumin
HSBP	Heparin sulphate binding protein
HSP	Heat-shock protein
H2RA	H2 receptor antagonist
IceA	Induced by contact with gastric epithelial cells gene A
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL-8	Interleukin 8
IM	Intestinal metaplasia
Le a	Lewis a
Le b	Lewis b
LF	Lactoferrin
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
mAb	Monoclonal antibody
MALT	Mucosal associated lymphoid tissue
MAPK	Mitogen activated protein kinase
Mb	Minibody
MBL	Mannose binding lectin
MHC	Major histocompatibility complex
MP	Mannoprotein

MW	Microwave
MWt	Molecular weight
NCTC	National Collection of Type Cultures
NLBH	Neuraminyl lactose binding haemagglutinin
OD	Optical density
ODU	Optical density units
Oip	Outer inflammatory protein A
OMP	Outer membrane protein
PAI	Pathogenicity island
PBP	Penicillin-binding protein
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline/Tween 20
PGC	Polyglycosylceramides
PE	Phosphatidylethanolamine
PI	Propidium Iodide
PIER	Proteolytic induced
PL	Poly-D-Lysine
PLP	Periodate-lysine-paraformaldehyde
PLPD	PLP dichromate
PMN	Polymorphonuclear leukocyte (Neutrophil)
PPI	Proton pump inhibitor
PRR	Pattern recognition receptor
PU	Peptic ulcer
PUD	Peptic ulcer disease
PL	Phospholipase
RGD	Arg-Glyc-Asp amino acid sequence
rRNA	Ribosomal RNA
RVC	Recurrent vaginal candidiasis
SabA	Sialic acid binding adhesin
Sap/*SAP*	Secreted aspartyl proteinase (protein and gene respectively)
sIgA	Serum IgA
scIgA	Secretory IgA
sLe x	sialyl-Lewis x
sLe a	sialyl-Lewis a

SSC	Side scatter
3'SL	3' sialyllactose (NeuAc2-3Gal1-4Glc)
TBS	Tris buffered saline
TBST	Tris buffered saline/Tween 20
TFF	Trefoil factor
TGF-β	Transforming growth factor beta
.TIF	Tagged image file format
TLR	Toll-like receptor
VacA	Vacuolating cytotoxin A
VC	Vaginal candidiasis
VEC	Vaginal epithelial cell
V_H	Variable domain of heavy chain
VHD	V_H dummy (ie. V_H against an irrelevant antigen)
V_K	Variable domain of Kappa-light chain
V_L	Variable domain of Lambda-light chain
VVC	Vulvovaginal candidiasis
WHO	World Health Organisation
WT	Wild type
ZSF	Zinc salts fixative

UNITS OF MEASUREMENT

µm	Micrometers	L	Litre
nm	Nanometres	ml	Millilitre
g	Gram	M	Molar
mg	Milligram	°C	Degrees centigrade
µg	Microgram	kDa	KiloDalton

SECTION I

INTRODUCTION

Chapter 1

GENERAL INTRODUCTION

1.1 Microbial Adhesion

Adhesion of an organism to its host tissue is the first step (and probably the most critical) in the initiation of microbial infection.[1-3] The event itself is a complex process involving highly specific molecular interactions rather than simply a random 'sticking' of the organism to its host.[4,5] The result of adhesion is an induction of phenotypic and behavioural changes in both the host cell and the adherent microorganism.[6,7] Once attached, many microorganisms subsequently invade the tissue where they either grow, reproduce or exit and become disseminated, reaching other bodily organs via the blood or lymphatic systems.[8]

1.1.1 Surfaces involved in microbial adhesion

Within a normal healthy individual, there are three main types of surface to which microorganisms can attach: the skin, teeth and mucosae. Within each type of surface subdivisions upon subdivisions exist, depending upon the anatomical and topographical site.[4,5] Organisms may attach directly to the epithelial surface or may bind to components of the extracellular matrix (ECM).[9-15] However, the external layer of many body surfaces is coated by host secretions (for example the mucosae secretes a thick aqueous mucus containing an assortment of salts and glycoproteins) thus preventing many microbes from attaching directly to the epithelial cell layer beneath.[16] The mucus, which is expelled at regular intervals, is a major host defence mechanism and removes with it many organisms that have become trapped within.[4,17] In order to successfully reach their target tissue, microorganisms must therefore cross the mucus barrier and adhere to the epithelial surface before they are expelled. Epithelial surfaces are also shed. However, although the turnover rate is rapid (cells die within a few days of being formed),[18] it is much slower than that of the mucus.[19] The vaginal and intestinal mucosae are different to most other shedding surfaces, because biofilms (consisting mainly of Clostridia, Bifidobacteria and Lactobacilli) are able to form on them.[5]

1.1.2 Mechanisms involved in microbial adhesion

Adherence of organisms to all types of surface is very similar from a molecular point of view. Several different types of interaction, occurring at different distances from the substratum, come into play as the organism approaches the surface **(Figure 1).** The first interactions that occur are Van der Waals forces, the result of dipole induction in the two objects, causing the two surfaces to attract. These forces operate when the organism is >50 nm from the surface.[5,20] When the organism moves in closer, at a distance of approximately 10-20 nm, the significant forces operating are primarily electrostatic.[5,21] Despite both organism and surface being negatively charged,[22,23] which would usually lead to repulsion, the highly ionic nature of most natural environments is enough to overcome the repulsive effect (repulsion is reduced with increasing ionic strength). Charged surfaces attract 'counterions' from the surrounding environment which form a double layer of diffuse ions at the cell surface.[5,24,25] As the microbe moves closer still to the surface, at a distance of about 0.5-2.0 nm, a third type of interaction occurs: hydrophobic interactions.[5] Water molecules in the environment usually obstruct contact between the two surfaces, however, since both organism and/or substratum are covered with hydrophobic molecules, water is repelled by the surfaces and excluded, enabling hydrophobic interactions between organism and substratum.[5,24,26] This allows the organism to become sufficiently close to the surface of the substratum (<1.0 nm distance) for more specific adhesive interactions to function, such as hydrogen bonding, cation-bridging and receptor-ligand interactions.

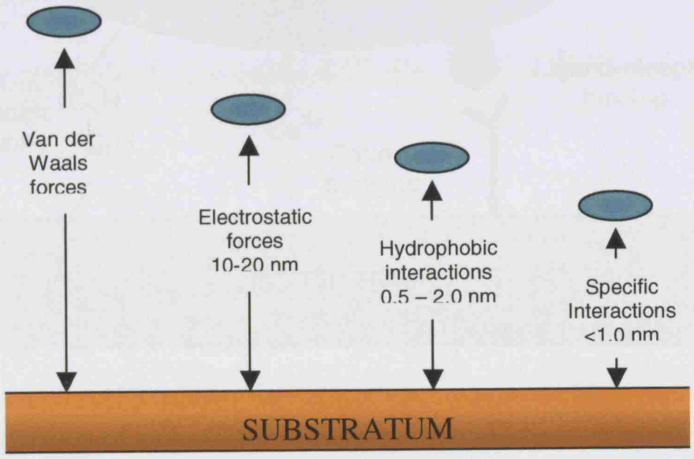

Figure 1: Physiochemical forces involved in microbial adhesion operating at different distances between the organism and substratum to which it adheres (after Wilson et al.[5])

Hydrophobic interactions, cation-bridging and receptor-ligand interactions are considered to be the primary forces involved in microbial adhesion to host surfaces[5] **(Figure 2)**.

1.1.2.1 Hydrophobic interactions

Hydrophobic bonding is generally thought to be a non-specific interaction because the occurrence of stereospecific interactions between the molecules involved is not evident.[27] The surface of a microbe or substratum is covered with hydrophobic molecules such as hydrocarbons, aromatic amino acids, fatty acids and mycolic acid, which are responsible for the hydrophobic interactions occurring during microbial adhesion.[5,28] Such molecules are known as hydrophobins and may also exist as non-polar regions on otherwise highly polar molecules such as carbohydrates and proteins. When a microbe approaches the surface of a substratum, intervening layers of water molecules become displaced by the presence of non-polar molecules on the surface of the organism and substratum being in close proximity.[5,24] Adhesion of the two surfaces results because of an energetically favourable event (due to the increased entropy which occurs as the water molecules are displaced).[5,29]

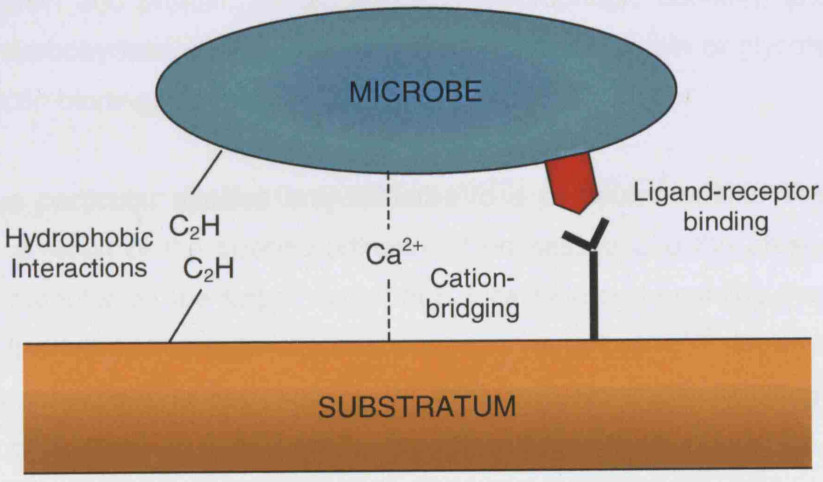

Figure 2: The primary types of molecular interaction involved in microbial adhesion (after Wilson et al.[5]).

1.1.2.2 Cation-bridging

Cation-bridging is important in the co-aggregation of organisms, adhesion of oral bacteria to tooth surfaces and between the negatively-charged molecules on host cell surfaces and microbes. As mentioned earlier, the overall net charge of the surfaces of microbes, inanimate objects and host cells is negative. Instead of repelling one another, these surfaces attract because divalent positive ions of metals such as calcium, (which are found in the surrounding aqueous environment), form a bridge between the two surfaces, thereby overcoming the repulsive force.[30-32]

1.1.2.3 Receptor-ligand binding

Microorganisms express cell surface adhesins, molecules which often have a high specificity for interacting with carbohydrate and protein structures expressed on the surfaces of eukaryotic cells, to which they form a strong, but non-covalent bond.[5] Host cell receptors, or ligands, are complementary in structure to the microbial adhesin and the interaction between the two is therefore highly specific. This specificity offers the opportunity to develop agents that inhibit this key interaction thus preventing the process of adhesion. Only a small and specific portion of the molecules involved are required for adhesion, and these are known as epitopes (or adhesiotope in the case of the receptor molecule).[3,5] Receptors or ligands comprise a variety of molecules such as proteins, polysaccharides, glycoproteins and glycolipids. Binding can occur between protein and protein, protein and lipid (hydrophobic bonding) and between protein and carbohydrate (which may be found on a glycoprotein or glycolipid) and is known as lectin binding; the lectin refers to the protein.[5,33]

Usually a particular species only adheres to a particular type of tissue (tissue tropism) as a result of the specific adhesins it possesses and the presence of the appropriate receptor on the target tissue; thus localising and enabling them to infect the specific tissues that carry these complementary receptors.[5,34,35] However, despite successfully adhering to its target tissue, the microbe faces another limiting factor: the appropriate environmental conditions for growth and reproduction must be present at the site in order for its continued survival.

1.1.3 Structures involved in adhesion

1.1.3.1 Microbial structures

Adhesins may be present on any part of the external surface of the microbe, in the case of bacteria this includes the capsule,[36] flagella[37] and cell wall.[38,39] For fungi, these adhesins may be cell wall components found particularly on the external fibrillar layer.[38,40-42] Microbes may also have specific structures that function solely for the purpose of adhesion, for example the fimbriae and pili of bacteria[43,44] or the fimbriae of fungi.[45] An individual organism may possess several different adhesins although they may not all be expressed simultaneously. This is an important feature of invasive organisms that encounter different surfaces and thus require different adhesins to attach at particular stages of the invasive process.[33,35] As a consequence of the organism's necessity to express adhesins for its survival, it has made itself susceptible to the host immune system, which will eventually produce secretory IgA (scIgA) antibodies against the adhesin, which bind to it and prevent the microbe from attaching to the host surface/tissue.[46-48] Some organisms have overcome this problem by implementing phase variation of antigenic structures.[49,50]

1.1.3.2 Host cell structures

Microorganisms are able to adhere to three main structures of host cells: Firstly they can bind directly to structural components of the lipid bilayer; [5,51] secondly they can bind to cell surface molecules that are receptors for host cell molecules[52] and thirdly they may bind indirectly to host molecules that are already bound to the cell surface, such as ECM components.[9] The typical lipid bilayer of a eukaryotic cell membrane contains intrinsic, transmembrane and extrinsic proteins, the latter two of which may contain the specific amino acid sequences (epitopes) or carry carbohydrate structures (glycoproteins), which serve as receptors for microbial antigens.[53] The lipids of the membrane itself such as phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, cholesterol as well as several glycolipids may also contain such epitopes.[5] Host cells often present receptors that are necessary for host cell molecules (such as hormones,[54-56] immunoglobulins,[57] cytokines[58] and ECM molecules[14,59]) that are involved in signalling or defence. These molecules have therefore become a prime target for the utilisation of organisms, some of which have developed complementary adhesins that bind to these structures.[35] Organisms also produce adhesins that can bind to structures within the ECM, such as laminin and

fibronectin. This is particularly important for organisms that have an extracellular existence such as *H. pylori*.[53] Adhesins responsible are known as MSCRAMMs (microbial surface components recognising adhesive matrix molecules).[60]

1.1.4 Consequences of adhesion

The process of adhesion not only involves the interaction between the receptor and ligand themselves but has physiochemical consequences for both the microbe and the host cell to which it has bound, which are brought about by molecular cross-talk (exchanging of signals) between organism and host.[61]

1.1.4.1 Effects on the microbe

Once adhered, the microorganism often adjusts phenotypically in order to survive in the new environment that surrounds it. This is brought about by up-regulation and suppression of numerous gene products, which often occur in response to changes in pH, nutrient concentration and osmolarity.[35] Production or expression of enzymes for new metabolic pathways may enable the organism to take advantage and utilise the host cell nutrients that are available to it in the new environment. For example, many bacteria produce siderophores that sequester iron from host lactoferrin molecules and iron is essential for the growth of many types of bacteria.[62,63]

Some changes, however, are brought about by adhesion itself. For example, upon adhesion secretion systems of particular microorganisms are activated, enabling them to inject proteins such as toxins into the host cell.[64,65] Because adhesion may be the preliminary step of cellular invasion by the microorganism, adhesion may act as a signal for the up-regulation and synthesis of molecules that are essential for invasion of the host cell or new adhesins so the microbe can adhere to new tissues that it encounters during invasion or dissemination.[35] Some bacteria produce substances that deactivate host cell defences, for example they may inhibit the synthesis or secretion of antibacterial peptides by epithelial cells.[66]

1.1.4.2 Effects on the host

Microbial interactions with epithelial cells most frequently involve members of the normal microflora, and they seem to have developed a commensalistic or mutualistic

relationship with the host cells to which they are attached.[67] Despite the fact that all bacteria are composed of molecules such as LPS (lipopolysaccharide), LTA (lipoteichoic acid) and peptidoglycan, which are potentially harmful to host cells, these molecules in normal microflora seem to have relatively little effect on host cells.[5,68-70] Commensals and pathogens both interact with host cells by molecular cross-talk, however, they trigger different responses from epithelial cells and immune cells.[72-74] It still remains to be determined exactly how the human body differentiates between pathogenic and commensalistic microorganisms and the current status of knowledge of commensal and pathogen-host interactions is the subject of two recent reviews by Kelly et al.[61,75]

Upon attachment, the host cell responds in several ways: some are defensive measures while other responses are the result of toxins or molecules produced by the adherent microorganism, which in many cases has detrimental effects on the host cell.[76,77] In order to defend itself against microbial infection, host cells may secrete antibacterial peptides (in the case of epithelial cells) to kill the adherent microbes,[78,79] or they may release cytokines to recruit and activate immune cells that subsequently kill or immobilise microorganisms.[80-82] Cells may also produce chemokines, which are sticky proteins and act as guides for immune cells to the site of infection as well as activating them.[80] These chemokines also cause the endothelial cells and immune cells to produce complementary intercellular adhesion molecules, which enable the immune cells to adhere to the endothelial lining of the blood vessel and then pass across (diapedesis) the capillary wall to the site of infection.[83-86] Epithelial cells may also phagocytose organisms once they have attached. Epithelial cells are not normally phagocytes, but some bacteria force their entry in epithelial cells by a virulence process called invasion.[8,87,88] Such bacteria produce adhesion molecules known as 'invasins',[89] which activate the cytoskeletal machinery of host cells inducing it to phagocytose the bacterium thus enabling it to enter the cell where it has a good supply of nutrients and is protected from host defences such as complement and antibodies.[5]

A common effect of adherent microorganisms on host cells is the induction of an altered morphology, forming an adhesion pedestal or an attaching and effacing lesion.[90] An attaching and effacing lesion occurs for example, when Enteropathogenic *E. coli* (EPEC) infects the epithelial cells of the intestine. Adherent *E. coli* inject proteins (for example Tir, Map, EspF and EspH)[91] into the host cell which undergo tyrosine phosphorylation and become anchored in the host cell membrane where they

act as a receptor for an adhesin of EPEC, known as intimin, which enables the organism to become intimately attached to the host cell.[92,93] This induces the rearrangement of cytoskeletal components and actin polymerisation, resulting in the loss of microvilli (effacing) and the production of an adhesion pedestal (attaching), an extension from the surface of the cell.[94,95] Moreover, toxins produced by adherent microorganisms (for example, Gliotoxin produced by fungi such as *C. albicans* and *Aspergillus*) may lead to fluid loss or even apoptosis of the host cell.[96-99]

1.1.5 Factors that affect adhesion

Growth phase of the microorganism for example *Candida* – stationary phase cells have increased adhesion to Hep-3 cells, which may be due to a higher concentration of adhesins that develop over time. Stationary cells also are more hydrophobic which aids adhesion.[100-103] Another factor affecting microbial adhesion is **growth temperature**. For example, *C. albicans* cells grown at 37°C have increased adherence compared with those grown at 25 and 28°C. This is because the organism has adapted to live in humans whose core body temperature is stabilised at 37°C.[104] **Nutritional status** of the organism is another factor affecting adherence. For example, it has been shown that *C. albicans* grown in sugar-rich media display higher levels of adhesion due to an increase in the production of a fibrillar-flocular layer on the outer cell surface.[105] An environment that is rich in sugars (for example the oral cavity of persons who consume large amounts of dietary sugar), would lead to increased virulence of the infecting yeast.[5] **Interactions with other members of the microflora** are known to have both a positive and negative effect on the adhesion of pathogenic microorganisms. The microflora may physically block adhesion of pathogens[106-108] or produce antimicrobial substances such as bacteriocin, which affect only the pathogenic microorganism.[109-111] However, adhesion of pathogens may be enhanced by the presence of microflora, for example for pathogenic fungi, the commensals may form the basis of a substrate to which the fungal cells can adhere or agglutinate and form a biofilm.[112,113]

1.2 Mucosal Infections

1.2.1 The mucosa

The epithelial surface of the body comprises all surfaces that are topologically exposed to the external environment. The surfaces exposed to the environment are the skin, the digestive tract running from mouth to anus, the respiratory and urogenital tracts.[5] There are two distinct types of epithelia: the 'dry' epithelium (namely the skin) and the 'wet' or 'mucosal' epithelium that encompasses all other epithelial surfaces. The mucosae are covered with mucus, which is a thick aqueous substance containing salts, proteoglycans and glycoproteins (mainly mucin), secreted by goblet cells that are interspersed amongst the epithelial cells.[114-116] Although they are multifunctional, the main role of the epithelial cells is to form a physical barrier separating the internal and external spaces of the body.[116]

Because epithelial surfaces are exposed to the environment, they have a high probability of encountering microorganisms and thus need to be protected from microbial infection. A major role of the mucosa is to form the first line of antimicrobial defence.[5,117]

1.2.2 Antimicrobial defence

The mucosa provides defence against microorganisms in three major ways: physically, chemically and biologically.

1.2.2.1 Physical defence

The inside of the body is maintained as a sterile environment because the epithelial surface forms an unbroken physical barrier to the outside.[116] Several physical mechanisms occur within the mucosal environment, which also restrict microbial entry and colonisation. Microbes are removed from the mucosal surfaces by the flow of fluids such as saliva, mucus, gastric juices, urine and tears; or by the action of hairs, cilia and mucus which cover several mucosal surfaces. They are consequently trapped and passed out of the body, out of harm's way.[116]

1.2.2.2 Chemical defence

An alternative strategy adopted by the body to combat microbial attack, is to maintain an environment where the pH is not conducive for microbial growth. The mucosa achieves this by producing a variety of chemicals that alter the pH of the area. For example, in the stomach, the mucosal cells (oxyntic cells) secrete hydrochloric acid, resulting in an environmental pH of 2.0, which kills most microorganisms. In the small intestine the pH is alkaline (due to bile secretion) resulting in conditions that very few organisms can grow in.[4] In the vagina the pH is maintained below neutral (at pH5.0) due to the production of lactic acid by members of the microflora such as lactobacilli.[118-119]

1.2.2.3 Biological defence

As well as secreting chemicals to prevent microbial growth, the mucosa also produces many specific biomolecules with antimicrobial effects. Interestingly, the normal microflora of the host does not seem to be susceptible to many of these defences and are able to maintain their colonies at the mucosal surfaces.[61,75]

a) Mucus - Mucus acts as a lubricant, traps microbes and provides a physical barrier preventing microbes form reaching the underlying epithelium. The mucins (complex glycoproteins) within are responsible for most of these properties.[120] Mucus also contains antimicrobial substances such as lysozyme,[121,122] peroxidase[123] and lactoperoxidase,[124] which are secreted by the epithelial cells. The antimicrobial substances of the mucus are active against bacteria and fungi.

b) Lysozyme – Lysozyme is found in sweat, tears, nasal secretions and is produced by mucosae in general. This enzyme cleaves the bond between N-acetylglucosamine and N-acetylmuramic acid in the peptidoglycan of bacterial cell walls.[125] It also can interefere with the activity of LPS, causing conformational changes which reduces the inflammatory response (inhibits cytokine release) thus rendering it less toxic[126,127] and can interact synergistically with other antibacterial molecules produced by epithelial cells (ECs) to enhance the overall antibacterial effect.[128] Lysozyme is active against bacteria, fungi and viruses.

c) Lactoferrin – Lactoferrin is a glycoprotein with a high binding affinity for iron. Because iron is an essential element required for life, both host and microbe compete

to obtain it; a strategy adopted by both organisms to kill each other.[5,129] Lactoferrin is produced by polymorphonuclear leukocytes (PMNs) and ECs and is present at the mucosal surface, which results in there being very little free iron available for microorganisms.[130] Apart from preventing microbial growth by sequestering iron, lactoferrin has antibacterial properties. It binds to LPS (which would stimulate an inflammatory immune response) thus inhibiting its activity,[131,132] blocks the binding of many bacteria to the EC surface,[133] interacts with components of the external cell wall[134-136] and co-operates with lysozyme as a microbicide since when cleaved, lactoferrin produces lactoferricin, a potent antimicrobial peptide.[137] Lactoferrin is effective against bacteria, fungi and viruses.

d) Lactoperoxidase – Lactoperoxidase is a glycoprotein produced by mucosal cells and its main function is thought to be antibacterial defence.[138] By-products of its catalysis are superoxide ions[139] and the production of various other antibacterial compounds, some of which inhibit microbial adhesion.[140-143] Lactoperoxidase is effective against bacteria, fungi and viruses.

e) Secretory phospholipase A_2 – Phospholipase A_2 is particularly effective against gram-positive bacteria, (the cell membrane of gram-negative bacteria is less exposed), because by removing fatty acids from phospholipids it is able to damage cell membranes.[79,144,145] So far, secretory phospholipase A_2 has only been shown to be effective against bacteria.

f) Secretory Leukocyte Protease Inhibitor (SLPI) – PMNs and ECs of the mucosa produce SLPI (a protease inhibitor) in response to the presence of bacterial LPS.[146,147] SLPI interacts with LPS and downregulates macrophage responses to the antigen thus protecting tissues from the detrimental effects of inflammation.[148] SLPI is active against viruses,[147] bacteria[148] and fungi including *C. albicans*.[150,151]

g) Trefoil peptides – These are peptides with a triple loop structure (hence the name trefoil) and are found closely associated with mucins because they are secreted and synthesised by mucin-producing ECs of the GI tract.[152,153] They have an indirect antimicrobial effect, because they are involved in the maintenance and repair of the mucosal epithelium.[154]

h) Acute phase proteins (APPs) – APPs are a specific type of pattern recognition molecule whose main function is to bind to microbial molecules (such as

phospholipids in bacteria or mannose and β-glucan in fungi)[155-157] and opsonise the microbes so that they are more easily phagocytosed and destroyed by cells of the immune system.[158-159] APPs are found in serum and are produced mainly by the liver but can also be produced by mucosal ECs.[160] APPs are active against bacteria and fungi.

i) Collectins – Collectins are proteins with multiple carbohydrate binding domains and are produced by most mucosal surfaces including the gut and liver.[161] They are thought to be an important part of the innate immune system, acting as a PRR (pattern recognition receptor) recognising bacteria, fungi and viruses.[159,162,163] Collectins include the serum protein mannose binding lectin, (MBL) and lung surfactant proteins A and D (SP-A and D).[164,165] By binding to carbohydrates on the surface of bacteria, bacterial cells are aggregated and can be cleared from the mucosal surface by immune cells or other defensive measures.[166] In the presence of SP-A and D, gram-positive and gram-negative bacteria have been shown to be more readily taken up by neutrophils suggesting collectins also have a role in opsonisation.[167] LPS-induced cytokine and nitric oxide production has also been shown to be inhibited by SP-A.[168]

j) Secretory IgA (scIgA) – The mucosa plays an important role in guarding the body's internal environment. In order to do this it has a close association with the lymph cell system, which activates B lymphocytes to produce IgA.[4,169] ScIgA is found in large amounts in the external mucosal surfaces (except for the genital tract) and these antibodies are thought to inhibit the adhesion of many bacteria to the mucosal surface.[57,170,171] This happens because IgA produced by the plasma cells in the submucosa is transferred through the EC cytosol and onto their surface. This process is known as transcytosis and is mediated by a specific receptor (transferrin).[172] ScIgA is active against bacteria, fungi and viruses.[173,174]

k) Antimicrobial peptides – The ECs of the mucosa actively produce antibiotic peptides (such as human α- and β-defensins) which bind to negatively charged organisms and form pores in, cause thinning of or destabilise microbial membranes.[175-178] They may also permeabilise microbial membranes by interfering with electrostatic charge.[179,180] Although most cells of the body produce antibacterial peptides, very few, like the mucosa and circulating phagocytes, produce them in large amounts.[5] Antimicrobial peptides are effective against bacteria, fungi and viruses.[181]

1.2.3 Microbial infection of the mucosa

Mucosal surfaces are commonly attacked by bacteria, fungi and viruses because they are constantly exposed to the outside environment. Two important pathogens of mucosal surfaces in humans are the fungus *C. albicans* and the bacteria *H. pylori*. Although the types of mucosal surfaces these microbes infect are very different, they have two major traits in common: both microbes are able to cause severe mucosal diseases and both are emerging as resistant organisms to current antibiotic therapy.[182-184] These microbes, the diseases they cause, their mechanisms of adhesion and the therapies used to treat infection are described in detail in the subsequent sections of this chapter.

1.3 *Candida albicans*

Organisms of the genus *Candida* are the most abundant pathogens causing fungal infections in humans.[185] The most virulent organism of this genus and one responsible for the majority of these diseases is *C. albicans*.[4] *C. albicans* poses no threat of disease in normal, healthy individuals, where it lives naturally as a commensal of the mucosal surfaces of the oral cavity, vagina and gastrointestinal tract of humans and animals. It is only under conditions that lead to host debilitation that infection occurs, which is why *C. albicans* is known as an 'opportunistic' organism.[4]

Candida albicans is a polymorphic yeast (**Figure 3),** forming several morphologically distinct phenotypes: yeast-like cells (also known as blastospores or blastoconidia) and long filamentous outgrowths known as hyphae and pseudohyphae.[4] Depending upon environmental conditions, *C. albicans* can reversibly switch from one form to another.[186,187] The parent cells are the blastospores, which are gram positive, spherical to subspherical unicellular cells, between 4-6μm in size.[188] True hyphae are outgrowths of the yeast cell and pseudohyphae often form from yeast cells during budding, when the cells remain attached to each other. The ability to form several different morphotypes (see phenotypic swiching 1.3.3.3) confers distinct virulence advantages upon the organism, enabling it to colonise many different locations within the host, which is why it is the cause of a very wide range of clinical manifestations of disease.

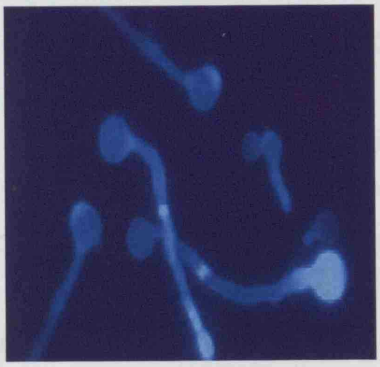

Figure 3: *C. albicans* - budding yeast cells, germ tubes and hyphae (from http//:www.reviberoammicl.com/photo-gallery/candida/albicans)

1.3.1 Diseases caused by *C. albicans*

There are over 154 species of *Candida*, six of which are most frequently isolated in human infections: *C. albicans, C. tropicalis, C. glabrata, C. parapsilosis, C. krusei* and *C. lusitaniae*; all of which are pathogenic.[189,190] *Candida* species are found worldwide and they are among the most common infectious agents in man.[191] The wide range of infections that are caused by organisms of the genus *Candida* are collectively referred to as candidiasis (candidosis). *C. albicans* in particular is responsible for the majority of these diseases, accounting for up to 70% of all *Candida* species isolated from sites of infection.[192]

In the absence of disease as many as 80% of people may be colonised by commensal *C. albicans*.[193-195] Conditions that lead to host debilitation and hence *C. albicans* infection include: cancer or immunocompromised patients, changes in the host physiology or normal microflora.[196-201] The greater the severity of the host's debilitation, the greater the seriousness of the disease that may result from *C. albicans* infection. Less virulent species do account for some infections, but in order for these organisms to invade, the host must be more severely impaired.[202]

There are two main categories of candidiasis: superficial and systemic.[188] Systemic (or disseminated) candidiasis is usually a nosocomial infection and is a rare disease except in severely debilitated patients. It can result from the continual introduction of yeasts into the body by repeated injections in drug users, indwelling

catheters or long-term therapy with antibiotics or steroids. Infection occurs throughout the body (spread via the blood stream) affecting multiple organ systems. In people with these conditions, the prospect of recovery is small and the death rate is high.[188,203] Superficial candidiasis consists of localised infections that may be cutaneous (affecting the skin and nails) or mucocutaneous (affecting the mouth, vagina, oesophagus or bronchii). Interestingly, the most common form of disease caused by *C. albicans* colonisation belongs to this category and is known as 'thrush' (oral or vaginal candidiasis).

1.3.1.1 Vaginal candidiasis

Approximately three-quarters of all women will suffer from vulvovaginal candidiasis (VVC) at least once in their lifetime and up to 25% of these women will experience recurrent disease (recurrent vulvovaginal candidiasis, RVVC), which is distressing and difficult to treat.[118,204,205] The most important cause of vaginal candidiasis is *C. albicans*; over 80% of infections are accounted for by this species alone.[189,206] The second most common species to be isolated from the genital tract of women with vaginitis is *C. glabrata*, which accounts for approximately 5% of infections.[188]

Many women harbour *C. albicans* in their genital tract and yet are asymptomatic for vulval or vaginal candidiasis. This suggests that in order for the yeast to exert its pathogenic effects, changes in the environment of the vagina are required. Uncontrolled diabetes mellitus (due to impaired neutrophil killing), antibiotic therapy, tight insulating clothing, HIV-infection/AIDS, use of oral contraceptives and pregnancy are all known to be predisposing factors.[202,207-211] Pregnant women suffer most from vaginal candidiasis, particularly during the third trimester, when at this time vaginal pH is at its lowest. A significant proportion of women who first have the condition when pregnant, afterwards suffer from either recurrent or chronic candidiasis.

The clinical symptoms of vaginal candidosis are intense itching (pruritis), swelling and burning of the vulva and vagina.[210] Onset is often abrupt and tends to commence the week before menstruation in women who are not pregnant. Erythema and fissuring of the vulva is the most common clinical manifestation and sometimes there can be vesicular lesions and inflammation of the vagina (vaginitis). In more serious cases pustules, excoriations and ulcers can form. The source of infection and reinfection of VVC was previously thought to be contamination from the digestive tract, however this is no longer thought to be true.[212]

1.3.2 Pathogenesis of Candida infection

The first step of infection is adhesion of *C. albicans* to the host tissue. This may be to epithelial or endothelial cells themselves or to components of the ECM. Once contact has been made, adhesion is facilitated by secreted enzymes which degrade and damage cell membranes and extracellular proteins, often exposing further potential receptors which the microorganism can bind to.[213,214] Adhesion to the host is a crucial event in the pathogenic process; it is required for colonisation and subsequent invasion or infection. Additionally, by adhering to the host, the yeast is able to resist forces such as the bathing/washing action of body fluids, which result in its removal from the body. Once adhered, hyphae may be produced which penetrate cell layers (assisted by enzymes which help degrade tissues) and this has the combined effect of allowing the yeast cells to enter into the host bloodstream. Hyphae use thigmotropism (contact-sensing) to find the path of least resistance through the layers of cells.[215] Within the blood stream, *C. albicans* utilises phenotypic switching or coats itself in platelets in order to evade the immune system.[25,216,217] It can also produce haemolysin to obtain the iron essential for growth from the erythrocytes that this protein has lysed.[218] *Candida* may leave the bloodstream to disseminate within various organs of the body and in order to do this it must first adhere to the endothelial lining of the capillary and pass through the vessel wall.

1.3.3 Virulence factors of *C. albicans*

The life of *C. albicans* as a commensal is still relatively unknown, which means that it is uncertain whether factors thought to be involved in the virulence of the organism are truly associated with pathogenesis or are in fact involved with its survival as a commensal. Virulence factors have been defined as components of pathogens that damage the host,[219] traits that are required to establish disease[220] and factors that directly interact with host cells.[221] If opportunistic pathogens are to establish infection, they must survive and replicate within the host environment, spread to new areas and at the same time evade host immune defences. In order to do this *C. albicans* possesses a number of virulence traits that have been implicated in its ability to cause disease. Many of the investigations which have led to the identification of these factors have been carried out on oral and systemic models of *Candida* infection, relatively few have looked at the virulence factors involved in VVC. Below is a brief summary of the general virulence factors thought to be involved in *Candida* infection.

1.3.3.1 Adhesion

Adhesion of *Candida* cells to the host tissue is one of the most critical initial steps in the pathogenesis of both superficial and systemic disease; cells which do not adhere are more likely to be removed from the host by physical and immune defences. In order for *C. albicans* to invade host cells, it must first adhere to them. Once adhered it produces invasins that force it to be taken up (phagocytosed) by host cells or alternatively it produces invasive hyphal outgrowths. *C. albicans* cells which lack genes encoding certain adhesins, have been shown to be less virulent *in vitro* and *in vivo*.[222-224] It has also been shown in animal models that non-adherent strains of *C. albicans* cause less disease.[225-228] Both the hyphal and yeast forms of *C. albicans* have been shown to be involved in adhesion.[229,230] A number of putative adhesins have been proposed for *C. albicans* and these will be discussed in more detail later on.

1.3.3.2 Morphogenesis

C. albicans is a polymorphic yeast and its ability to reversibly convert from yeast to hyphal form is thought to be important for virulence.[231,232] Most infected tissues contain both growth forms and each has a different role in virulence. It has generally been shown that the yeast form is involved mainly in adherence of the organism to host tissue, whereas the hyphal form is involved in both adherence and post-adhesion tissue invasion.[233-235] Additionally, it has recently been shown that septin proteins, not hyphal growth alone, are required for invasion.[236] Hyphae have been shown to play an important role in virulence. Strains which are unable to form hyphae have reduced virulence or are avirulent *in vitro* and *in vivo*, including in vaginal infection.[237-241] Hyphae as well as yeast cells have been shown to be involved in adhesion, in particular to vaginal epithelial cells (VECs) and hyphal growth has been found to favour adherence.[242,243] The enhanced adherence ability of hyphal forms is thought to be attributable to its increased cell surface hydrophobicity (CSH).[244]

The ability to form two different distinct morphological forms (yeast and hyphae) confers distinct advantages on the pathogenic potential of the microorganism. These include:

1. Aiding evasion of the immune system

Different morphotypes of *C. albicans* have been shown to be able to modify the differentiation pathways of the immune system in different ways, which may be a contributing factor to the organism's ability to evade the immune system (for example, evasion of phagocytosis by hyphae and the release of different cytokines).[245,246] Both the yeast and pseudohyphal forms of *C. albicans* have been shown to be important in immune evasion because they have an attenuated proinflammatory phenotype compared to hyphae and it is the hyphal form of *C. albicans* that is essential for host killing.[247-249]

2. Aiding survival in the presence of the microflora

Interactions with the microflora are also different depending on *C. albicans'* morphological form. For example, the hyphal form but not yeast, has been shown to be sensitive to killing by members of the microflora such as *Pseudomonas aeruginosa*.[250]

3. Invasion

Following yeast cell adhesion, the hyphal form was shown to be a requirement for invasion.[7,234,235,250,251] Invasion, which occurrs after adhesion, is probably accompanied by the interconversion of yeast and filamentous forms (phase transition) as well as the secretion of enzymes which aid penetration of the tissues by the organism.[253,254] The adherence and invasion phenotypes are closely correlated with morphogenesis.[7]

4. Other advantages

The ability of *C. albicans* to form germ tubes also seems to enhance its virulence potential. When germ tubes form, new surface antigens are expressed,[255-258] the cell has increased CSH[259-261] and enhanced expression or induction of germ-tube specific receptor molecules.[262-265] All these changes allow *C. albicans* to bind to a range of tissue types which is important for establishing infection and reducing susceptibility to host defence, particularly in disseminated candidiasis.[229]

1.3.3.3 Phenotypic switching

C. albicans colonies are able to swich between a number of different phenotypes. Phenotypic variations may be morphological, including changes in cell shape (round, elongated or bean-shaped); colony colour (white or opaque/grey) and colony texture

(smooth, rough, wrinkled, fuzzy or stippled depending on differences in cell-surface structures).[266-267] White colonies tend to be smooth, comprising round-ovoid cells and opaque colonies are usually flat and grey, consisting of elongated or bean-shaped cells. In an animal model of systemic infection, white cells were found to be more virulent than opaque cells.[268] The studies of Soll and Srikantha[269] have shown that strains of *C. albicans* freshly isolated from vaginitis or systemically-infected patients, display high frequencies of phenotypic switching. It is not known, however, whether this is the result of genome variation rather than a relationship to the development of disease.

Although phenotypic switching has been demonstrated in *C. albicans*, and some links with virulence have been shown, the mechanism and involvement of phenotypic switching in virulence of the organism is still not clear. It is likely, as suggested by Cotter and Kavanagh,[25] that in addition to a change in the actual phenotype, switching alters other factors such as anti-fungal drug resistance, adhesins and the production of extracellular enzymes. In fact it has been shown that opaque cells specifically express the genes for secretory aspartyl proteinases 1 and 3 (*SAP1* and *SAP3)* while *SAP2* is expressed by white cells. Phenotypic switching allows the organism to exploit multiple different microniches within the body of the host.

1.3.3.4 Secretion of enzymes

Two main types of proteinase have been implicated in the virulence of *C. albicans*: Secreted aspartyl proteinases (Saps) and Phospholipases (PL). Proteases disrupt host mucosal proteins and allow the invasion of mucosal surfaces by the growing *C. albicans* hyphae. A protease-negative strain has been shown to be avirulent compared to its revertant.[270] A strong correlation has been found between proteolysis *in vitro* and active symptomatic vaginitis.[271]

Secreted aspartyl proteinases

So far, ten different *C. albicans* Saps have been identified.[272] Saps are able to degrade many human proteins at the sites of lesions, for example albumin, keratin, mucin, complement, lactoferrin, lactoperoxidase and secretory IgA and IgG.[273-278] The proteolytic activity of Saps has been shown to be associated with tissue invasion[279] thus enabling the organism to penetrate the tissue barrier and invade. Degradation of the ECM by Saps also allows *C. albicans* to gain access to nutrients.[38] Differential expression of Saps has been shown for the two different morphs of *C. albicans in*

vitro: Saps 1, 2 and 3 are mainly expressed in yeast cells whereas expression of Saps 4-6 is confined to the hyphal form.[280-281] The proteolytic activity of Saps means that they also have a role in adhesion; by degrading the mucin that covers mucosal surfaces, the underlying epithelial cells are exposed for the microorganism to adhere to.

The genes for Saps 1-8 have been tested for their *in vivo* expression in human oral and vaginal *Candida* infection. *SAPs* 1, 3 4, 7 and 8 were shown to be correlated with oral disease and *SAPs* 1, 3, 6 - 8 were correlated with vaginal disease.[282] In experimental vaginal candidiasis (reconstituted human vaginal epithelium), Schaller and co-workers[283] found that *SAPs* 1 and 2 were responsible for tissue damage whereas *SAPs* 3, 4 and 6 were not. They also showed that during the progression of infection, different *SAPs* were detected (*SAPs* 2, 9 and 10 initially then 1,4 and 5 followed by 6 and 7 at the later stages of infection. In a rat model of vaginal *Candida* infection the product of *SAP2* was required for development of disease.[284] The discrepancies between studies showing which Saps are involved in vaginal infection (i.e. Sap2 was not detected in human infection) are probably due to differences in the models used (human and animals).

Phospholipases

There are four main types of phospholipase (A-D), classified according to the specific ester bond of glycerophospholipid molecules which they are able to cleave. Phospholipase B is responsible for the major phospholipase activity in *C. albicans*.[285,286] Phospholipases have been shown to be linked to the virulence of *C. albicans* infection, for example, in a murine model of disseminated *Candida* infection cells with reduced secretion of phospholipases were found to be less virulent than strains producing large amounts.[285] Phospholipase B1 was shown to be required for virulence in a murine model of disseminated infection[287,288] and is detected in the hyphal tips during tissue invasion.[289]

Lipases

Very few studies have investigated secreted hydrolytic lipases and esterases in *C. albicans* infection and therefore little is known of their role and function. These enzymes catalyse the hydrolysis of the ester bonds found within mono- and poly-glycerolipids and phospholipids. Most genes for lipases are detected at the yeast-hyphal transition and since there are a large number of genes encoding these

enzymes, they are thought to be important in the persistence and virulence of *C. albicans* in a number of different tissues.[213]

1.3.3.5 Other virulence traits

Adaptations for survival within the vagina

pH and CO_2 levels have been shown to affect adhesion of *C. albicans* to the vagina.[290] The environment of the vagina is maintained at a very low pH (approximately pH5.0), due to the production of lactic acid by members of the microflora, such as lactobacilli.[118] *C. albicans* has adapted to live in the low pH environment of the vaginal canal, the low pH itself is a factor which is known to often promote/aid *C. albicans* proliferation and growth.[202,291] For example, the PHR1 gene associated with cell wall synthesis (operating usually around a neutral pH) is switched off and a second pH-regulated gene PHR2 operates with a similar function at acidic pH.[231,232]

C. albicans usually causes infection of the vagina during the luteal stage of the menstrual cycle or during pregnancy, when levels of oestrogen are high. Infection occurs mainly at these times because elevated oestrogen enhances several factors that are required by *C. albicans* to colonise the vaginal epithelium. Firstly, it has been shown to reduce the ability of VECs to inhibit *C. albicans* growth.[292-294] Secondly, glycogen levels are increased which can be a source of nutrition for the fungus and thirdly, epithelial cells become keratinised; a factor known to make the epithelial cells more susceptible to *C. albicans* adhesion.[292,295-301] *C. albicans* is also able to survive in high salt concentrations which have been shown not to affect its growth or adherence properties.[302]

To summarise, although several traits are involved in the virulence of *C. albicans* infection, it is the combined functioning of all the virulence factors together that result in its ability to cause disease. Despite the operation of virulence factors, ultimately, changes in the host environment and immune system are required for microorganisms such as *C. albicans* to be able to change from a commensalistic to pathogenic lifestyle. However, of all the virulence traits a key factor for survival is the ability of the microorganism to adhere.

1.3.4 Adhesion in *C. albicans*

Like most other microbial cells, the external surfaces of fungi are covered with a thick, multi-layered cell wall overlying the cell plasma membrane.[4] The structure of the *C. albicans* cell wall has been described by Shepherd[303] (**Figure 4**). The cell wall contains at least three major polysaccharides: chitin, glucan and mannoprotein (MP) of which the former two are key integral structural components. MPs are glycoproteins that are composed of a mannan oligosaccharide (which contains the adhesion epitopes of *C. albicans*) linked covalently to protein by glycosidic bonds.[38] The degree of MP glycosylation is variable and differences in expression depend upon the age of the cell and the growth form (e.g. blastoconidia or hyphal). MPs are not only found within the cell wall but are also located at the outer surface.[38] The outermost (fibrillar) layer of the yeast-cell wall is composed of mannoproteins and it is this layer which is believed to contain the fungal adhesins and receptors.[40,304] In addition to cell wall components, specialised structures such as fimbriae have been shown to be involved in adhesion of *C. albicans* to host cells.[305,306]

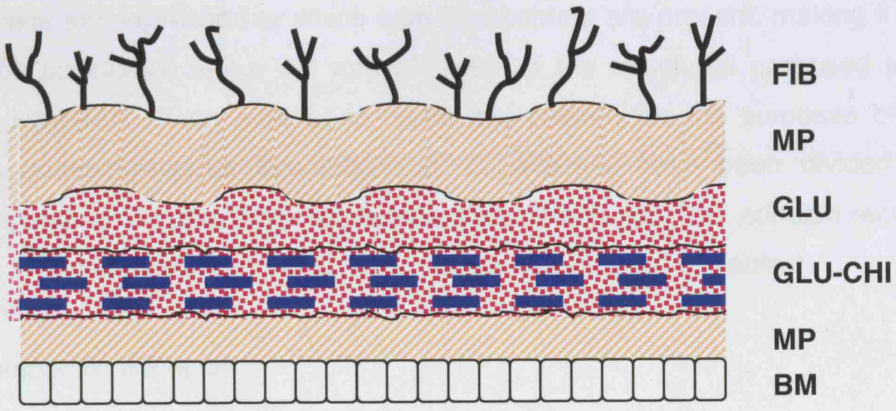

Figure 4: Schematic diagram of *C. albicans* cell envelope (after Calderone et al.[225] GLU = β-glucan; FIB = fibrillar layer; GLU-CHI = β-glucan-chitin layer, MP = mannoprotein; BM = basement membrane.

Because *C. albicans* is able to infect a wide range of niches within the human host, it expresses multiple adhesins which enable it to attach to many different types of structure including epithelial cells, endothelial cells and components of the ECM. Adhesion to such surfaces is mediated through specific adhesin-receptor interactions as well as non-specific interactions, of which the cell surface hydrophobicity of the organism plays an important role.[307,308] Cell surface hydrophobicity is particularly important in the adhesion of *C. albicans* to inert structures such as plastic catheters, which are inserted into hospital patients and are therefore often the major source of nosocomial *C. albicans* infection.[309,310]

A number of adhesins have been described for *C. albicans.* Most of the studies investigating *C. albicans* adhesin-receptor interactions have been performed on endothelial cells or components of the ECM. Those investigating adhesion to the mucosa are usually performed on buccal epithelial cells (BECs), while only a handful have involved the vagina. The following review of *C. albicans* adhesin-receptor interactions therefore includes general *C. albicans* adhesins as well as adhesion to mucosal epithelial cells. Specific work done using the vagina is mentioned.

Despite the numerous studies investigating *C. albicans* adhesin-receptor interactions, very little work has been done to delineate the specific epitopes of either the adhesin or the receptor involved. Limitations of studies investigating adhesin-receptor interactions include: large variations in the methods employed and the type of tissues, strain and growth media used. Taken together, all of these factors affect which adhesins are expressed or which type of receptors are present, making it very difficult to be conclusive about the role of some of the structures proposed to be involved in adhesion. In an attempt to make this clearer, for the purposes of this review, the adhesin-receptor interactions of *C. albicans* have been divided into sections depending on the type of surface they attach to. The adhesin-receptor interactions involved in adhesion of *C. albicans* are summarised in **Table 1**.

1.3.4.1 Non-specific adhesion

The two major non-specific adherence interactions involved in *C. albicans* adhesion are electrostatic interactions (as described previously) and cell surface hydrophobicity (CSH). Non-specific interactions are involved in the adhesion of *C. albicans* to the ECM, endothelial and epithelial cells as well as to inert materials such as plastic.[100,308,311,312] It is thought that the CSH of *C. albicans* results from the numerous

lipids and mannoproteins present in its cell wall (see **Table 1**) and cells which exhibit a higher degree of hydrophobicity have been shown to have a greater adherence ability to plastic, epithelial cells and ECM proteins.[309,311,313-315]

CSH has also been shown to be involved in the pathogenicity of the organism. For example, since CSH has been shown to precede yeast to hyphal conversion, it has been suggested that as a commensal *C. albicans* cells are hydrophilic and that the yeast converts to a hydrophobic form when it becomes pathogenic.[103,314,316,317] This is important since it enhances adhesion and resistance to phagocytosis.[318-320] Pseudohyphae or hyphae have been shown to be typically more adhesive than blastospores of the same strain,[321,322] which is probably due to the higher CSH of the filamentous form. The CSH of yeast cells is affected by temperature, nutrition and growth stage.[322] Variation in CSH has been shown to be brought about by changes in the structure and conformation of the fibrillar layer which is a function of growth phase.[314,324]

1.3.4.2 Specific adhesion

C. albicans uses specific adhesins to adhere to the ECM, endothelial and epithelial cells of host tissue. Virtually all identified *C. albicans* adhesins have been shown to be proteinaceous in nature and most of them are mannoproteins.

1.3.4.2.1 Adhesion to endothelial cells and the ECM

Adhesion to endothelial cells and the sub-endothelial ECM is mainly a requirement for disseminated candidiasis.[325] The interactions between *C. albicans* and the ECM and endothelial cells are outlined in **Table 1**.

The main proteins within the endothelial ECM that *C. albicans* is thought to target include: Fibronectins (high molecular weight glycoproteins involved in cell adhesion and migration; located in the interstitium of the ECM); Collagen Types I and IV (found in the interstitium and basement membrane) and Laminin (also found in the basement membrane). Klotz et al.[326] have shown that *C. albicans* is able to bind to all of these molecules when they are immobilised on plastic surfaces.

Mammalian cells including endothelial cells, secrete and display C3d and iC3b-binding proteins on their surface[327] and are known as complement receptors (CR2 and CR3 respectively). Since human CR3-like proteins are found on the blastospores,

germ-tubes and pseudohyphae of *C. albicans*, they may be important adhesins involved in *C. albicans* adhesion to endothelial cells.[328] Adherence of *Candida* species to endothelial cells has been shown to be correlated with the expression of CR3-like receptor molecules.[329-331] More recently adhesion of *C. albicans* yeast and hyphal forms to endothelial cells was shown to be mediated by human C4b binding protein (C4BP), a complement (classical pathway) inhibitor. Surface-bound C4BP serves multiple functions including immune evasion (inhibits complement activation at the yeast surface) and is involved in the adhesion of *C. albicans* to endothelial cells.[230]

1.3.4.2.2 Adhesion to epithelial cells

Adhesion to epithelial cells is important for *C. albicans* infection of mucosal surfaces. Although numerous investigations have looked at *C. albicans* adhesion to epithelial cells and a number of putative adhesin-receptor interactions have been proposed, to date many of the interactions have not yet been fully defined; elements of either the adhesin or receptor are unknown.

Most investigations into the adhesion of *C. albicans* to human epithelial cells, seem to support the notion that mannoproteins have a major role. It is thought that the protein-portion of the mannoprotein in particular is responsible for adhesion. Adhesion via mannoproteins is thought to be strain-specific[332] and it has been shown that there are significant antigenic differences between the cell wall mannoproteins of different *C. albicans* isolates and different *Candida* species (for example degree of glycosylation).[333] It has been suggested that this may be due to colonial phenotypic switching which is known to affect antigenic characteristics of the cell surface[266] and may be associated with changes in glycosylation in mannoproteins as shown by Martinéz et al.[324]

The numerous range of adhesins for *C. albicans* reflects the number and types of different tissue the organism is able to infect. CSH is also important in adhesion, probably forming the initial step of a two-step process and as the organism gets nearer the host surface, specific interactions come into operation.

<u>To summarise</u>

Four main systems seem to have been identified so far which are used by *C. albicans* for adhesion to host surfaces (after Calderone[335]). It remains to be determined

whether adhesion is based on four different mannoproteins or different components of the same one. Of these four systems, two have been identified for *C. albicans* adhesion to epithelial cell glycosides (System I and II).

System I: *C. albicans* mannoprotein with lectin-like properties recognises glycosides which contain fucosyl or *N*-acetylglucosamine on human buccal or vaginal ECs. This adhesin is present in the outer fibrillar layer of blastoconidia and has not been associated with hyphae yet. Studies have shown that the ligand-binding domain lies within the protein portion of the MP and it contains 65-82% CHO (mostly mannose) and approx 7% protein.

System II: Integrin-like proteins C3d- or iC3b binding proteins (complement receptors CR2 and CR3 respectively) found on *C. albicans* (fewer are found on blastoconidia than hyphae). They are MPs recognising RGD sequences of epithelial cells, ECM components or endothelial cells. C3d binding proteins have been purified and vary between 60, 68, 50 and 130-kDa MPs. It may be that various species have a single protein but they differ in degrees of glycosylation.

System III: A mannan oligosaccharide (probably the factor 6 epitope, a hepatomannosyl side-chain) is a proposed adhesin recognising EC receptors. This is different to the system I adhesin.

System IV: chitin and chitin-degradation products have been shown to be involved in adhesion of *C. albicans* to plastic, BECs, VECs and in the protection of mice from vaginal infection.

Colonisation of oral and other mucosal surfaces may be associated with the System I adhesin, normal or damaged endothelial cells with System II.

1.3.4.2.3 Adhesion to the vaginal mucosa

Relatively few studies have looked at the adhesion of *C. albicans* to the vaginal mucosa and study results are somewhat conflicting; probably due to the use of different methods and means of quantification. It generally seems however, that components of chitin and mannoproteins are the adhesin epitopes and probably recognise glycosides on the VEC surface.[336-339] A mannoprotein adhesin (in the outer

fibrillar layer of blastoconidia) with lectin-like properties has been shown to recognise glycosides containing fucosyl or *N*-acetylglucosamine on human VECs.[12]

Interestingly, VECs on the luminal surface have been shown to have complex surface ridge patterns (linear, circular, hooked) which are important for the adherence of *C. albicans* blastospores.[290] It has been shown that the ridged sides of VECs lead to greater adhesion of *C. albicans*.[290] Ridges are also important for thigmotropism of hyphae to aid penetration and invasion of weakened areas of the epithelial cell surface.[215] No studies in the literature seem to have looked at hyphal penetration at these sites or hyphal response to VEC topography.

Table 1: Adhesin-receptor interactions involved in *C. albicans* adhesion.

Calb = *C. albicans*, RGD = Arg-Gly-Asp amino acid sequence, MP = Mannoprotein, SAP = secreted aspartyl proteinase, CR = complement receptor, CSH = cell surface hydrophobicity, ALS = agglutin-like sequence, Hwp = hyphal wall protein, BECs = buccal epithelial cells, VECs = vaginal epithelial cells, ECM = extracellular matrix, PEG = polyethylene glycol, GAPDH = glyceraldehydes 3-phosphate dehydrogenase, RHE = reconstituted human epithelium, C3d and iC3b = complement fragments. Matrigel is a basement membrane preparation.

ADHESIN	ADHESIN EPITOPE	RECEPTOR	RECEPTOR EPITOPE	CANDIDA MORPH PRESENT ON	TYPE OF INTERACTION	TISSUE USED TO TEST	TYPE OF INFECTION INVOLVED	REFERENCES
Non-Specific adhesion								
Cell-surface hydrophobicity (CSH)	CSH caused by lipids and mannoproteins, chitin and hydrophilic fibrils	Inert materials (e.g plastic); epithelial and endothelial cells; ECM	n/a	Yeast, hyphae and germ tubes	Non-specific, hydrophobic	Epithelial cells, endothelial cells, ECM proteins, and plastic	Disseminated candidiasis; denture stomatitis	100, 103,261, 308, 311-313, 340, 341
Specific adhesion								
Extracellular matrix								
Integrin analogue; glycoprotein	Possibly β-1 Integrin subset	Fibronectin	RGD sequence of amino acids (Arg-Gly-Asp)	Yeast cells and germ tubes	Protein-protein	Adhesion of Calb to fibronectin	Disseminated candidiasis	25, 59, 342
37-kDa laminin-binding protein	Possibly β-1 Integrin subset	Laminin	Possibly B- chain peptide of laminin	Germ tubes and some yeast cells	Protein-protein	Binding of Calb to laminin	Disseminated and superficial candidiasis	25, 343-345
Integrin analogue	αβ-3-like subset	Vitronectin and endothelial cells	RGD sequence of amino acids (Arg-Gly-Asp)	Germ tubes and yeast cells	Protein-protein	Adhesion-inhibition of Calb to vitronectin and endothelial cells; binding of anti-Integrin Abs to Calb	Disseminated candidiasis	345, 346

Table 1 continued

ADHESIN	ADHESIN EPITOPE	RECEPTOR	RECEPTOR EPITOPE	CANDIDA MORPH PRESENT ON	TYPE OF INTERACTION	TISSUE USED TO TEST	TYPE OF INFECTION INVOLVED	REFERENCES
Extracellular matrix (continued)								
Integrin analogue	Subset unknown	Collagen (Type I)	RGD sequence of amino acids (Arg-Gly-Asp)	Yeast cells	Protein-protein	Adhesion to immobilised collagen	Disseminated candidiasis	347
58-kDa Mannoprotein (MP58)	O-glycosylated epitope	Fibrinogen	Unknown	Yeast cells and pseudohyphae	Unknown	BECs; adhesin binding to fibrinogen	Oral and disseminated candidiasis	265, 343, 348
Glycoprotein (60-kDa)	Unknown	Fibronectin, Vitronectin and Collagen (Type I)	Unknown	Yeast cells	Unknown	Adhesion of glycoprotein to fibronectin, vitronectin and Collagen	Disseminated candidiasis	13
22, 44 and 65-kDa protein	Unknown	Entactin (glycoprotein)	RGD sequence of amino acids (Arg-Gly-Asp)	Yeast cells and hyphae	Protein-protein	Adhesion of Calb to entactin	Disseminated candidiasis	229
60-68-kDa proteins; C3d-like binding proteins	Unknown	Fibrinogen, fibronectin and laminin	Unknown	Mainly germ tubes and hyphae; small amount on some yeast cells	Unknown	Adherence of Calb to ECM proteins; receptor purified	Disseminated and superficial candidiasis	33, 225, 262, 263, 349-351
Hydrophobic proteins	Unknown	Fibronectin and laminin	Unknown	Yeast cells	Unknown	ECM components and endothelial cells	Disseminated candidiasis	100, 352, 353
Several different adhesins; maybe glycosphingolipid binding adhesin of Cameron and Douglas (1996)	Unknown	Collagen Type IV	Oligosaccharides (mannose, fucose and GlcNAc) in the 7S domain of Type IV Collagen	Yeast cells	Lectin	Adhesion-inhibition of Calb to immobilised Type IV domain; Inhibition-adhesion of Calb to Hep2 cells by anti-Collagen IV mAb	Collagen IV is a basement membrane protein so probably disseminated candidiasis	354, 355
GAPDH (glyceraldehyde 3-phospate dehydrogenase)	Unknown	Fibronectin and laminin	Unknown	Yeast cells and germ tubes	Unknown	Inhibition-adhesion of Calb to immobilised ECM proteins; expression of GAPDH in clinical isolates	Disseminated candidiasis	356, 357
Unknown	Unknown	Tenascin-C (Multimeric protein of fibronectin, fibrinogen and other ECM components)	RGD-independent; Fibronectin Type III NOT laminin	Germ tubes NOT yeast cells	Unknown	Adhesion of Calb to soluble human Tenascin-C	Disseminated candidiasis	358

Table 1 continued

ADHESIN	ADHESIN EPITOPE	RECEPTOR	RECEPTOR EPITOPE	CANDIDA MORPH PRESENT ON	TYPE OF INTERACTION	TISSUE USED TO TEST	TYPE OF INFECTION INVOLVED	REFERENCES
Endothelial cells								
Unknown	Unknown	Fibronectin	Fn/20 and Fn/23 (20 and 23-mer peptides from fibronectin) RGD sequence	Yeast cells	Unknown	Yeast cells adhesion and inhibition to ECM proteins or PEG beads coated with ECM proteins	Disseminated candidiasis	359, 360
Complement receptors; proteins homologous to CR2 and CR3 members of β-2 Integrin family	Receptors for C3d and iC3b	C3d and iC3b (complement fragments)	Unknown	Yeast cells, germ tubes, hyphae. Pseudohyphae possess both receptors but yeasts do not.	Protein-protein	Inhibition-adhesion of Calb to endothelial cells; clearance of infection by iC3b receptor in mice	Disseminated candidiasis	329-331
CR2-like	50-kDa MP (Yeast cells); 60-kDa (hyphae); 62 and 70-kDa proteins; 55-115-kDa MPs (epitope not within oligosaccharide component)	C3d	Unknown	Yeast cells, germ tubes, hyphae	Protein-protein	Inhibition of rosetting by the MPs; binding of MPs to mAbs against iC3d binding protein (CR2 receptor); Inhibition of Calb adhesion to endothelial cells by MPs; Inhibition of Calb adhesion to iC3d by MPs	Disseminated candidiasis	304, 361, 362
CR3-like (β-2 Integrin); proteinaceous and likely to be glycosylated	42-kDa glycoprotein; or 165-kDa and 51-71-kDa mannoprotein	iC3b	Unknown	Yeast cells, germ tubes, hyphae	Unknown	Endothelial cells	Disseminated candidiasis	304, 323, 328
?? Ionic interaction thus maybe MPs or β-glucan	Unknown	PLASMA PROTEINS: Human C4-binding protein (C4BP); factor H and FLH1 (inhibitors of complement alternate pathway)	In N-terminal complement control protein domains (CCPs) of the α-chain	Yeast cells and hyphae	Unknown	Calb adherence to C4BP	Endothelial cells; disseminated candidiasis	230
Epithelial cells								
Lectin MANNOPROTEINS	Serotype A strain oligosaccharide of Factor/ Antigen 6 (pentaose or hexaose of mannan moiety)	Glycolipids	Oligosaccharides containing L-fucose, *N*-Acglucosamine, *N*-Acgalactosamine or lactose residues	Unknown	Lectin	BECs, human carcinoma and endothelial cells	Oral and disseminated candidiasis	323, 364, 365

Table 1 continued

ADHESIN	ADHESIN EPITOPE	RECEPTOR	RECEPTOR EPITOPE	CANDIDA MORPH PRESENT ON	TYPE OF INTERACTION	TISSUE USED TO TEST	TYPE OF INFECTION INVOLVED	REFERENCES
Epithelial cells (continued)								
MANNOPROTEINS	Mannose-containing moieties (e.g. α-methyl-mannoside). Mannan or MP as intact molecule required	Unknown	Unknown	Yeast cells and germ tubes	Unknown	Human BECs, heart valves, matrigel and fibrin-plated matrix, *in vitro* murine systemic infection	Oral, disseminated and systemic candidiasis	366-370
MANNOPROTEINS	NOT mannose or α-methyl-mannoside	Unknown	Unknown	Yeast cells	Unknown	VECs	Vaginal candidiasis	371
MANNOPROTEINS	Unknown	Unknown	Unknown	Yeast cells	Unknown	Uroepithelial cells and BECs	Oral and urinary candidiasis	372
MANNOPROTEINS	Protein portion	H-blood group antigen	L-fucose	Yeast cells; budding yeast cells	Lectin	BECs	Oral candidiasis	373, 374
MANNOPROTEINS (Glycoproteins containing mainly D-mannose) within fimbrial adhesin	PAK (128-144-kDa peptide)	Glycosphingolipids	Disaccharide component βGalNac(1,4)βGal of asialo-GM1	Unknown	Lectin	BECs and purified adhesin binding to asialo-GM1	Oral candidiasis	46, 305, 306, 375
Lectin-like MANNOPROTEINS	Protein portion	H antigen (glycoside)	Contains D-galactose, *N*-Ac-D-galactosamine, *N*-Ac-D-glucosamine and an α-1,2-fucose (the H sugar sequence in all ABO blood group structures)	Yeast cells and germ tubes	Lectin and Protein-protein	Keratinocytes from foreskin; BECs	Oral and penile candidiasis	342, 376
Two Lectin MP adhesins	Protein portion	Glycosides	L-fucose and *N*-Ac-D-glucosamine	Yeast cells	Lectin	BECs and VECs	Vaginal and oral candidiasis	12
Hwp1 MANNOPROTEIN (Hyphae specific protein)	Protein portion	Transglutaminase	??	hyphae	Unknown	BECs	Oral candidiasis	377

Table 1 continued

ADHESIN	ADHESIN EPITOPE	RECEPTOR	RECEPTOR EPITOPE	CANDIDA MORPH PRESENT ON	TYPE OF INTERACTION	TISSUE USED TO TEST	TYPE OF INFECTION INVOLVED	REFERENCES
Epithelial cells (continued)								
Chitin and MP	Amino sugars (mannose, mannosamine and glucosamine), chitin, chitin derivatives and constituents (e.g. *N*-acetyl-glucosamine)	Unknown	Unknown	Yeasts	Unknown	Inhibition-adhesion of Calb to GI tract by saccharides and reduced spread of Calb infection in mice *in vivo*; Calb cell wall components adhesion to VECs	Vaginal and disseminated candidiasis	337, 378
Chitin and chitin-degradation products (N-Ac-glucosamine, glucosamine and mannose)	Unknown	Unknown	Unknown	Unknown	Maybe interferes with CSH??	Chitin soluble extract reduced vaginal candidiasis *In vivo* in mice; human VECs and BECs	Vaginal and oral candidiasis	337, 339, 379
SAPs (in particular 1, 2, 9 and 10)	Unknown	Protein	Unknown	Yeast cells	Protein-protein	Glass surfaces, BECs and reconstituted human vaginal epithelium (RHVE); Matrigel	Vaginal, oral and disseminated candidiasis	283, 380-382
Enolase	Unknown	Unknown	Unknown	Yeast cells and germ tubes	Unknown	Epithelial and endothelial cells; Inhibition-adhesion of Calb by mAb against enolase to polystrene, BECs and Hep2 cells (laryngeal carcinoma)	Oral and disseminated candidiasis	383-386
Alcohol dehydrogenase and aryl alcohol dehydrogenase	CSH1p protein; Integrin-like proteins 37 and 38-kDa (α-β-1 and α-β-3)	Fibronectin and vitronectin	Unknown	Yeast cells	Unknown	Abs to integrins react with Calb alcohol dehydrogenase and block adhesion to fibronectin, vaginal and disseminated models of candidiasis	Vaginal and disseminated candidiasis	387-389
Cell surface factor 4 (extracellular glycosidase)	Unknown	Unknown	Unknown	Yeast cells	Unknown	Mammalian cells and *in vivo* mouse model of disseminated candidiasis	Disseminated candidiasis	390

Table 1 continued

ADHESIN	ADHESIN EPITOPE	RECEPTOR	RECEPTOR EPITOPE	CANDIDA MORPH PRESENT ON	TYPE OF INTERACTION	TISSUE USED TO TEST	TYPE OF INFECTION INVOLVED	REFERENCES
Epithelial cells (continued)								
ALS glycoproteins (Agglutin-like sequence)	Als1 and Als1p - binding epitope in N-terminus of Als1p	Degenerate peptide sequences present in most proteins	Unknown	Yeast cells and germ tubes	Unknown	Endothelial cells, RHE (Oral) and disseminated infection *in vivo* in mice	Oral and disseminated candidiasis	391-394
ALS glycoproteins (Agglutin-like sequence)	Als2p	Unknown	Unknown	Yeast cells	Unknown	Endothelial cells, oral RHE and BECs	Oral and disseminated candidiasis	395
ALS glycoproteins (Agglutin-like sequence)	Als3	Unknown	Unknown	Yeast cells and germ tubes	Unknown	Endothelial cells, BECs and RHE (Oral) NOT fibronectin	Oral and disseminated candidiasis	391
ALS glycoproteins (Agglutin-like sequence)	Als4p	Unknown	Unknown	Yeast cells	Unknown	Endothelial cells NOT other cell types (e.g. BECs)	Disseminated candidiasis	391, 395
ALS glycoproteins (Agglutin-like sequence)	Als5p (similar to Hsp70)	Degenerate peptide sequences present in most proteins (including fibronectin, laminin, Type IV collagen, BSA)	Patches of threonine, serine and alanine residues; peptide backbone and amino acid side chain	Yeast cells, germ tubes and pseudohyphae	Protein-protein	*In vivo* vaginal candidiasis; Calb adhesion to beads coated with peptides or ECM components	Vaginal and disseminated candidiasis	393, 396, 397
Phospholipids, sterols and steryl esters	Unknown	Unknown	Unknown	Yeast Cells	Unknown	BECs	Oral candidiasis	398
EAP1 gene	Unknown	Unknown	Unknown	Yeast cells and pseudohyphae	Unknown	Polystyrene and kidney epithelial cells	Disseminated candidiasis	399
CaNOT5 gene / URA3 gene (encodes orotidine 5' monophosphate decarboxylase enzyme)	Unknown	Unknown	Unknown	Yeast cells and hyphae	Unknown	Human BECs and murine disseminated candidiasis	Oral and disseminated candidiasis	400-402
Unknown	Unknown	Lactoferrin	Unknown	Yeast cells	Unknown	Vaginal monolayer	Vaginal candidiasis	403

Table 1 continued

ADHESIN	ADHESIN EPITOPE	RECEPTOR	RECEPTOR EPITOPE	CANDIDA MORPH PRESENT ON	TYPE OF INTERACTION	TISSUE USED TO TEST	TYPE OF INFECTION INVOLVED	REFERENCES
Epithelial cells (continued)								
2 types of adhesin	Unknown	Glycosides	L fucose, N-acetyl-D-glucosamine, D-mannose, D-glucosamine	Unknown	Lectin	VECs and BECs	Vaginal and oral candidiasis	12
Unknown	Unknown	Glycosphingolipids (lactosylceramide)	lactosyl and galactosyl residues	Yeast cells	Unknown	Lung cells and erythrocytes; binding to glycosides on overlay assay	Disseminated candidiasis	404
Unknown	Unknown	Blood group glycosphingolipids	N-Acetyl glucosamine	Unknown	Unknown	Chromatogram overlay assay	Unknown	374
Mucin and saliva								
Probably MP	Unknown	Salivary and serum proteins including fibrinogen, entactin	Oligosaccharide	Unknown	Unknown	Calb adhesion to immobilised fibronectin, mucin and salivary proteins	Oral and disseminated candidiasis	405
Unknown	Unknown	Salivary mucins (acidic fraction)	Heparan sulfate side-chain	Yeast cells	Unknown	Adhesion of Calb to saliva constituents on membrane filters	Oral candidiasis	406, 407
Unknown - Not Mannoproteins	Unknown	Mucin	66-kDa cleavage product of 118-kDa C-terminal glycopeptide	Yeast cells	Hydrophobic interactions	Small intestine mucin and BECs	Oral and disseminated candidiasis	408
Unknown	Unknown	Salivary proteins (proline-rich)	Unknown	Unknown	Unknown	Calb overlay on salivary proteins on nitrocellulose membrane	Oral candidiasis	409
Miscellaneous								
Oestrogen-binding proteins (46-kDa subunit)	Unknown	Steroid hormones eg. Oestrogen	Unknown	Yeast cells	Unknown	Purified oestrogen protein binding to Calb cell wall extracts	Disseminated candidiasis	54-56, 410
Unknown	Unknown	Unknown	Fucose	Hyphae	Unknown	Calb binding to fucose	Unknown	411

1.3.5 Consequences of *C. albicans* adhesion

Adherence to host cells is the first step of the internalisation process (in which *C. albicans* is taken up into host cells) and may occur through receptor-mediated endocytosis, which has been shown to occur with bacteria in urinary tract infections.[412] Drago et al.[413] have shown that VECs (and BECs), which are non-typical phagocytic cells, are able to phagocytose *C. albicans.* Once inside the cell the yeast is protected from antimycotic drugs, which do not easily penetrate cells. In systemic candidiasis, passage across the endothelial layer is considered an essential step for organ invasion.

After adhesion of the organism to the host cell, new genes are upregulated or transcribed leading to the synthesis of new proteins and the induction of signalling pathways as a result of protein phosphorylation.[6] New proteins are necessary for the conversion of yeast cells to hyphae, which are required for invasion, for the synthesis of new cytoskeletal wall during growth and for the production of secreted enzymes for invasion.[255,256,414,415] These changes all occur in order for *C. albicans* to be able to adapt to its new environment, to avoid the host immune system and to aid invasion of host cells.

Another post-adhesion event shown to occur in *C. albicans* is thigmotropism (contact sensing).[6, 215,416] Thigmotropism aids *C. albicans* invasion and the dissemination of infection since it is able to 'feel' its way around or penetrate host cells in order to reach the basal cellular layers and subsequently enter into the blood-stream. It also enables it to locate sites of weakened integrity where the hyphae can penetrate. In order for this to occur, changes in growth direction are required which would most likely be brought about by the upregulation of genes or new gene transcription, for example for the synthesis of new cytoskeletal wall. Such events are often controlled by signal transduction events for example, via mitogen activated protein kinase (MAP kinase) pathway.[237]

1.3.6 Host defences against *C. albicans*

A major consequence of *C. albicans* adhesion is the activation of host defences against the organism. Many glycolytic enzymes have been identified as immunogens during candidiasis and enolase is among them.[384-386] For opportunistic pathogens such as *C. albicans*, normal host defences are usually sufficient to prevent infection

and often allow such pathogens to exist as commensals. However, hosts may become susceptible to *C. albicans* infection because of several major reasons, the most important of these being the loss of phagocytic cells or T-cell defects (particularly involving neutrophils), which are usually the result of immunodeficiency.[417,418] Components of the immune system shown to be involved in defence against *C. albicans* include: phagocytes; secretory immunoglobulins; human β-defensins; complement and the production of antifungals such as activated lactoferrin and arachidonic acid by host cells.

Phagocytes (including macrophages) have been shown to be involved in the clearance of *C. albicans* from the site of infection.[419,420] Professional phagocytes such as dendritic cells (DCs) have the function of killing *C. albicans* and presenting it to the adaptive immune system. However, these cells have been shown to be less effective at intracellular killing and damaging *C. albicans* hyphae and blastoconidia compared to monocytes and macrophages. This suggests that it is the innate immune system that has the prime task of killing *C. albicans* whereas DCs are more important for antigen-presentation and initiating the adaptive immune system.[421]

One of main mucosal defence systems against microbial attack is the production of secretory IgAs (scIgAs). ScIgA and IgG have been shown to inhibit *C. albicans* adhesion to a variety of surfaces.[422,423] However, the activity of these antibodies against *C. albicans* is limited since *C. albicans* produces Saps which extensively degrade IgAs and IgGs,[278,424,425] thus favouring adherence of the organism, which is a major factor in its pathogenesis. Interestingly, Saps have optimal activity at low pHs which is therefore of particular importance in the vagina where they may assist *C. albicans* colonisation.

Human β-defensins –1, -2 and –3 have been shown to participate in immune responses against *C. albicans*. They are thought to work by disrupting the fungal membrane since adherence to oral epithelial cells by several strains has been shown to be inhibited by recombinant β-defensins.[426]

C. albicans has been shown to activate complement by all three types of activation pathway[230,427,428] which leads to its opsonisation and to anaphylaxis. Complement has been shown *in vitro* to decrease fungal growth and increase phagocytosis by PMN leukocytes and thereby reduce adhesion and the release of Saps.[429] However, *C. albicans* may evade the immune system by binding to platelets and fibronectin, which camouflage it from immune recognition.[25,217]

In response to *C. albicans* infection, host cells may produce several antifungal agents such as arachidonic acid, which has been shown to be released from host cells and to modulate the growth, morphogenesis and invasiveness of *C. albicans*.[185] A second antifungal agent produced by the host is activated lactoferrin, which has been shown *in vitro* to detach and block *C. albicans* adhesion to VECs.[403]

Immunity against *Candida* at mucosal sites is usually associated with T helper (Th) 1-type $CD4^+$ T-cells and it was previously thought that defective or dysfunctional Th-1 $CD4^+$ T-cells resulted in the development of VVC.[430] However, it has recently been shown that involvement of systemic or local T-cell-mediated immunity is lacking in *Candida* infection of the vagina.[430] Instead, data suggest that symptomatic VVC (i.e. *Candida* infection coupled with inflammation) is associated with an aggressive response by polymorphonuclear neutrophils (PMN) and an inflammatory response, although PMN seem to be ineffective at killing the organism in the vagina.[431,432] Whereas an innate non-inflammatory response appears to have an important role in protection against VVC (*Candida* infection is present but symptoms of inflammation are absent).[430]

The lack of local or systemic immune protection is proposed to be an immunoregulatory adaptation of the commensal relationship between host and fungus, in order to avoid recurrent inflammatory responses occurring at a reproductive site.[430,433,434] Studies in animals have shown that immunoregulatory tolerance of *Candida* infection may be mediated by γ/δ T-cells and the immunoregulatory cytokine known as transforming growth factor-β (TGF-β).[435,436] Additionally, there is some evidence that VECs themselves have a static effect on *Candida* cells and may play an important role in the innate host-defence against the organism.[293]

It was previously thought that Candida-specific cell-mediated immunity was the predominant host defence mechanism against *Candida* infection and that susceptibility to infection was associated with Th-2 type responses (i.e. antibody response) and resistance to infection characterised by Th-1 type responses.[437] It now seems therefore, that it is the innate immune system (i.e. inflammatory response) that is responsible for generating the most important response and this innate response has a two-fold role, both promoting and protecting against symptomatic VVC. However, it must not be forgotten that the development of VVC is dependent upon a number of factors: the predisposition of the individual host; the virulence properties of the infecting fungal strain and environmental conditions; all of which may alter the delicate balance between yeast and host.

To summarise, it appears that the normal host immune system is generally effective against *Candida* infection, keeping it at bay and allowing its presence as a commensal. When defects occur in the host immune system, *C. albicans* is able to become pathogenic. PMN infiltrate resulting in inflammation and the development of symptomatic infection, but are unable to kill the *Candida* cells. The ineffective host responses combined with *C. albicans* mechanisms to evade the immune system lead to the development of infection.

1.4 *Helicobacter pylori*

Helicobacter pylori is a gram-negative, spiral-shaped, microaerophilic bacterium (**Figure 5**), which colonises the stomach of over half the human population, meaning that it is probably one of the most common human pathogens.[438] The prevalence of infection, however, does vary from population to population and is especially highest in developing countries where approximately 80-90% of adults are infected compared to less than 40% in developed countries.[439,440] Infection is usually acquired in childhood and is transmitted from person to person either oro-orally or faeco-orally.[441,442] Although most infected people are asymptomatic, in 10-20% of those infected, persistent *H. pylori* infection is the cause of severe gastroduodenal diseases,[443] including peptic ulcers (gastric and duodenal ulcers), adenocarcinomas and gastric lymphomas such as MALT Lymphoma.[444] If left untreated, infection is usually life-long.[445] The success of the organism can be attributed to the multiple virulence factors that it possesses which allow it to be suitably adapted to survival within the harsh environment of the stomach.

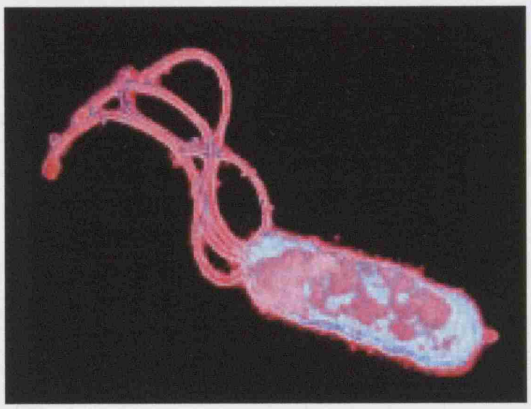

Figure 5: *H. pylori* (from the internet).

1.4.1 Survival in the stomach (virulence factors)

1.4.1.1 Acid and Urease

The first obstacle to survival in the human stomach is the highly acidic environment of the lumen. To overcome this problem *H. pylori* synthesises a cytosolic Ni^{2+} -containing enzyme, urease, which hydrolyses the urea present in the stomach, to produce ammonia and carbon dioxide,[444] (**Figure 6**). Urea is taken up through a proton-gated channel on the *H. pylori* inner membrane and once inside the cytosol the ammonia generated by the action of urease buffers (raises the pH of) the cytosol and periplasm.[446,447] Urease is also found on the outer surface of the bacterium[448] and the production of ammonia from the action of both the cytosolic and surface-bound enzymes creates a neutral layer around the bacterial surface.[449] Urease is an essential virulence factor for the bacterium; strains of *H. pylori* that are urease-deficient are unable to colonise the stomach.[450] Urea channels are regulated by the presence of protons, at acidic pH values they open to allow entry of urea to create the ammonia-buffer, but when the surrounding pH is neutral, they close to avoid over-alkalinisation, which is toxic to the bacterium.[451] Ammonia is also taken-up by *H. pylori* where it enters the nitrogen metabolic cycle and ultimately becomes incorporated into proteins.[452]

$$C{=}O(NH_2)2 + H^+ + 2H_2O \xrightarrow{\text{Urease}} HCO_3^- + 2(NH_4^+)$$

Figure 6: Hydrolysis of urea by urease; generating bicarbonate ions and ammonia.

Urease may also cause damage to host cells. *In vitro*, the ammonia it produces is toxic and membrane-permeable, causing alterations within cells such as swelling of acidic intracellular compartments, vesicular membrane transport alterations, depression of ATP production and protein synthesis and cell cycle arrest. It can also interact with metabolites of neutrophils such as myeloperoxidase, forming carcinogenic agents which may be involved in the development of *H. pylori*-induced adenocarcinoma[444,453-455]. *In vivo* however, the toxicity of ammonia produced by *H. pylori* is unclear because circulation of the extracellular fluids within the stomach may

sufficiently dilute it to below critical concentrations. However, it has been shown in rats that ammonia reduces the viability of mucosal cells by impairing mitochondrial and cellular respiration.[456, 457]

1.4.1.2 Mucus, flagella and enzymes

Once inside the stomach, it is necessary for *H. pylori* to leave the lumen because despite its acid-adaptation, it is not an acidophile. Additionally, if it remains within the lumen it will be cleared away by the mechanical movements and flow of fluids through the stomach. *H. pylori* therefore swims to the mucus layer that covers the epithelial lining of the stomach and propels itself through the mucus like a screw, by means of its helicoidal shape and the action of its polar flagella. It is then able to attach to the underlying epithelial cells and avoid acid exposure and host clearance mechanisms. Interestingly, non-motile mutants of *H. pylori* have been shown to be unable to colonise the stomach.[458] To aid its passage through the mucus layer, *H. pylori* also secretes mucus-degrading enzymes such as Phospholipases A_2 and C. These enzymes are also able to digest the phospholipid bilayer of the epithelial cell membrane thus releasing arachidonic acid, which is converted to compounds that may change membrane permeability and discharge of mucus, for example prostaglandins and leukotrienes.[459,463]

1.4.1.3 Adhesion

Another important step in the pathogenesis of *H. pylori* infection is the ability of the microorganism to adhere to the gastric mucosa, thus it avoids removal from the stomach by the mechanical action of host defences: peristalsis, gastric emptying and the continuous shedding and regeneration of the mucus layer. *H. pylori* adhesion will be discussed in more detail later on.

1.4.1.4 Pathogenicity Island and Type IV secretion system

Many bacteria contain pathogenicity islands (PAI) within their genome; these are groups of genes that encode specific virulence factors. For example, *H. pylori* possess a PAI known as the cagPAI comprising 30-40 genes that code for the CagA protein and its secretion system. The cagPAI is thought to have been originally acquired by horizontal transfer (from an unknown source) and divides *H. pylori* into Type I and Type II strains,[464] which will be discussed in more detail later on.

Many Gram-negative bacteria including *H. pylori*, possess elaborate secretion systems that are able to inject bacterial molecules such as proteins or DNA, directly into host cells. *H. pylori* possess a type IV secretion system, one of the two system types (types III and IV) which form molecular syringes.[465,466] The *H. pylori* type IV secretion system is an adaptation derived from the conjugative pili and is made up of the cag (cytotoxin-associated gene) system of proteins. *H. pylori* strains are classified into those that are *cag+* or *cag-*, reflecting the presence or absence of the cagPAI in the chromosome.[464] Approximately 12 genes of the cagPAI encode the proteins that form the building blocks of the *H. pylori* type IV secretion apparatus,[464] some of which comprise a molecular needle which is inserted into the host cell plasma membrane allowing the injection of bacterial proteins into the cytosol of the host cell..

1.4.1.5 Cytotoxins - CagA and VacA

H. pylori produces two known cytotoxins, the CagA (cytotoxin-associated gene A) and VacA (vacuolating cytotoxin A) proteins, which cause damage to host epithelial cells and are involved in stimulating an immune response to infection, which causes further damage to the gastric mucosa.[467]

CagA and CagE

CagA is a 128 to 145-kDa protein encoded by the *cagA* gene of the cagPAI and acts as a marker of this PAI. The *cagA* gene is not found in all strains of *H. pylori*; approximately one half to two-thirds of western isolates are c*agA* positive whereas in East Asian populations nearly all strains are *cagA* positive.[468,469] Almost all *cagA* positive strains have been shown to produce the CagA protein,[470,471] which is injected into epithelial cells where it undergoes tyrosine phosphorylation[76,472] and activates a number of signalling pathways.

These signals have multiple effects on host epithelial cells for example, inducing the dephosphorylation of cellular proteins such as cortactin and the rearrangement of the actin cytoskeleton of the host cell. This results in the formation of adhesion pedestals, the 'hummingbird' phenotype (cell elongation formed by the colocalisation of cortactin and filamentous actin in the tip and base of cell projections), cell spreading, migration, scattering and adhesion.[473-476] Signalling effects may also lead to cell proliferation and apoptosis,[477,478] which increase the risk of developing gastric cancer. Interestingly, anti-apoptosis (slowing the turnover rate of epithelial cells) has

also been shown to result from CagA phosphorylation, and is thought to aid the persistence of *H. pylori* attachment to the mucosal surface.[479]

CagA is also a highly immunogenic molecule and is the target of multiple components of the host immune response to *H. pylori* infection.[467] In particular, the cagPAI is involved in stimulating the gastric epithelium to produce pro-inflammatory cytokines (e.g. IL-8) and its presence in western populations has been associated with more severe *H. pylori*-induced disease.[480-482]

The *cagE* gene is also located within the cagPAI and is another marker of the PAI. CagE has been shown to induce the production of several cytokines from infected epithelial cells[483] and has also been shown to induce apoptosis in parietal cells.[484] *H. pylori* strains containing cagE have been found to be associated with the presence of DUD in children.[485]

VacA

A second cytotoxic protein known as vacuolating cytotoxin A (VacA) is produced by *H. pylori* cells. The *vacA* gene which codes for the cytotoxin is highly conserved and is present in all *H. pylori* strains, however a number of polymorphisms exist.[486] The most notable function of VacA, as the name suggests, is the induction of large intracellular vacuoles within gastric epithelial cells.[487,488] VacA reacts with ATPase (the enzyme that regulates membrane proton pumps), which is present on late endosomes inside the cell. Endosomes are therefore prevented from entering their usual endocytic cycle and instead fuse with each other. The proton pump is stimulated by the toxin and creates an acidic environment within the vacuoles. Basic substances such as ammonia pass across the vacuole membrane and are protonated in the acidic environment, rendering them hydrophilic and no longer able to pass back across the vacuole membrane. These molecules build up within the vacuole and lead to a change in osmotic potential so that water enters the vacuole and it swells[489-492]. It has been shown *in vitro* that over a period of two days, vacuoles fuse with each other, increase in number and size and eventually the cell membrane ruptures and lyses, killing the cell.[493]

The VacA protein itself, once secreted, is also able to bind to gastric ECs and form pores in the cellular membranes, which enables anions and urea to leak out,[494,495] the urea of which can be hydrolysed by *H. pylori* to aid its survival in the acidic gastric environment. Other effects of VacA include: loosening of tight junctions

between GECs;[496] immune suppression (blocks phagosome maturation,[497] inhibits T-cell proliferation and antigen presentation[498] and downregulates Th1 effects by blocking signalling);[499] decreased mucin synthesis[500] and lowering acid secretion by parietal cells.[501] *In vitro*, VacA has also been shown to induce apoptosis, suppress epithelial cell migration and proliferation and induce cytoskeletal changes.[502,505] Most of the effects leading to cell damage probably aid delivery of nutrients to the bacterium for its continued growth and survival within the stomach.[506]

The *vacA* gene has two regions (signal and m regions (**Figure 7**), which vary significantly in sequence, in different *H. pylori* strains. The s (signal) region is located at the 5' end of the gene and the m-region in the middle. There are two main s-region types: s1 and s2; and three known s1-region sub-types: s1a, s1b and s1c. The m-region also has two major types, m1 and m2. Despite the fact that all strains of *H. pylori* possess the *vacA* gene, variation among strains occurs in their ability to actually produce the cytotoxin. According to the studies of Atherton et al. and others[77,486,507,508] type m1 strains are more toxigenic than m2 and s1a strains more active than s1b. Type s2 seem to produce no detectable activity.

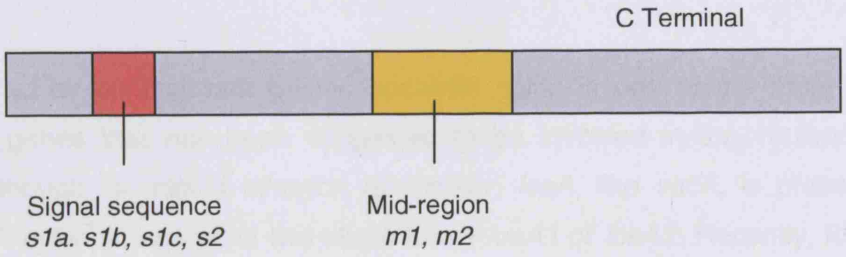

Figure 7: Schematic representation of the diversity in the *vacA* gene [based on diagram and information in Atherton et al.[486,470]

As mentioned previously, *H. pylori* strains can be divided into two main groups depending on the virulence genes they possess[509] (**Table 2**). Type I strains possess the cagPAI and express the cagA protein. They also carry the *vacA* gene and secrete active VacA cytotoxin. Type II strains have no cagPAI and carry the vacA gene but do not produce the active cytotoxin (**Table 2**). It is generally recognised that Type I strains are more highly toxigenic than Type II strains.[510,511] *CagA+* strains have been

shown to be associated with a significantly increased risk of developing severe gastritis, atrophic gastritis, PUD and distal gastric cancer, compared with *cagA*-strains[512-516] and strains with vacuolating activity are more commonly found in patients with PUD and gastric cancer than those patients with only superficial gastritis.[486,517-519]

	Genome		Protein	
	cagA	***vacA***	**CagA**	**VacA**
Type I	+	+	+	+
Type II	-	+	-	-

Table 2: Differences between Type I and Type II *H. pylori* strains

1.4.1.6 Other virulence genes

Ice A

IceA (induced by contact with gastric epithelial cells) is one of the more recently discovered genes that has been suggested to be involved in the virulence of *H. pylori*,[520] although its role in infection is unclear. *IceA*, like *vacA*, is present in all strains of *H. pylori*, in one of its two allelic forms *iceA1* or *iceA2*. Recently, *iceA1* has been shown to be associated with gastritis[521] and with more serious diseases when present in combination with other virulence genes such as *vacA* and *cagA*.[517,522]

Hp-NAP

H. pylori-neutrophil activating protein (otherwise known as Hp-NAP), is a 150,000 kDa protein[523] whose main function is to induce the adhesion of neutrophils to endothelial cells[524,525] and their recruitment by transendothelial migration to the site of infection, [526] which it does by binding to receptors on the neutrophil cell surface.[527] It is therefore important for the continuous recruitment of neutrophils to the gastric mucosa of *H. pylori* infected patients. It also activates neutrophils, monocytes and mast cells to produce reactive oxygen intermediates and it induces an antibody response. Hp-NAP

is therefore an important immunogenic molecule.[527] Hp-NAP may also be secreted or found as an OMP on the surface of *H. pylori* where it has a secondary function as an *H. pylori* adhesin, binding to receptors that are not found on neutrophils.[528]

LPS

H. pylori LPS (lipopolysaccharide) contains *O*-specific chains which hold identical antigenic epitopes to the human Lewis x or Lewis y blood group antigens.[529,530] Lewis x or Lewis y are found on cells of normal gastric mucosa.[531,532] It has been suggested these antigens expressed by *H. pylori* play a role in molecular mimicry, camouflaging the bacterium from being detected as a 'foreign' organism,[533-535] but this has been shown to cause stress to the host leading to the production of autoantibodies.[536] Consequently, Monteiro et al.[537] have suggested that these molecules may lead to targeting of the bacterium to particular sites within the gastric mucosa. The O-chain of LPS itself is able to directly induce the immune system leading to gastritis and gastric damage.[538] LPS has also been shown to decrease mucin synthesis.[539] It has recently been shown that phase variation in LPS prevents surfactant protein D (SP-D) binding to it (which is a molecule of the innate immune system and aggregates *H. pylori*) thus LPS aids the establishment of infection.[540]

1.4.2 Diseases caused by *H. pylori*

50-80% of the world's population are infected by *H. pylori,* and 10-20% of these are symptomatic.[443,541] The current incidences of *H. pylori* infection in diseases: chronic active gastritis 71-94%; gastric ulcer 72-100%; gastroduodenal ulcer 73-100% and a high prevalence in MALT Lymphoma disease since treatment for *H. pylori* results in a high regression rate (approximately 75%) of the MALT Lymphoma.[542-550] The diseases associated with *H. pylori* infection are shown in **Figure 8.**

The human stomach is divided into five different topographical regions, which vary in their composition of cell types **(Figure 9).** The entire lining of the stomach is made up of epithelium containing mucus-secreting glands, however, the types of glands present are different in each region. The upper part of the stomach comprises the cardia, fundus and body regions, of which, the fundus and body contain acid-secreting parietal cells, chief cells (which produce pepsinogen) and endocrine cells that secrete histamine, serotonin and somatostatin. The antral region of the lower stomach also contains endocrine cells, which secretes gastrin, serotonin and somatostatin.[551]

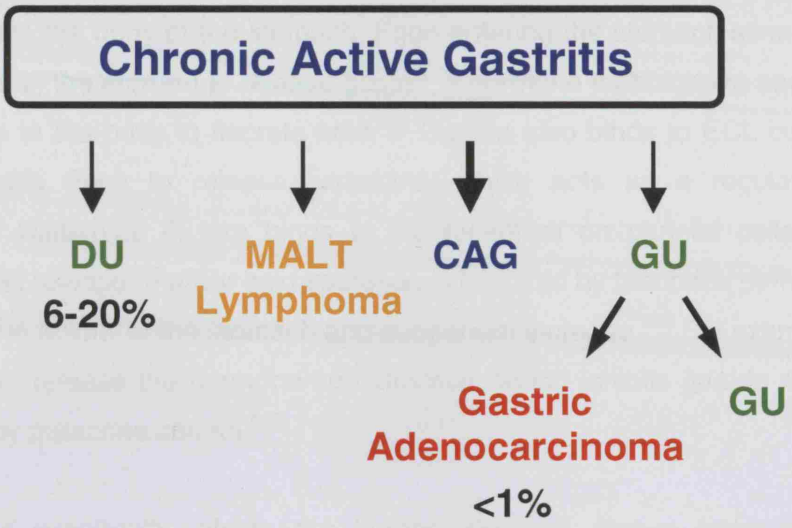

Figure 8: Diseases associated with *H. pylori* infection. CAG = chronic active gastritis; DU= duodenal ulcer; GU = gastric ulcer.

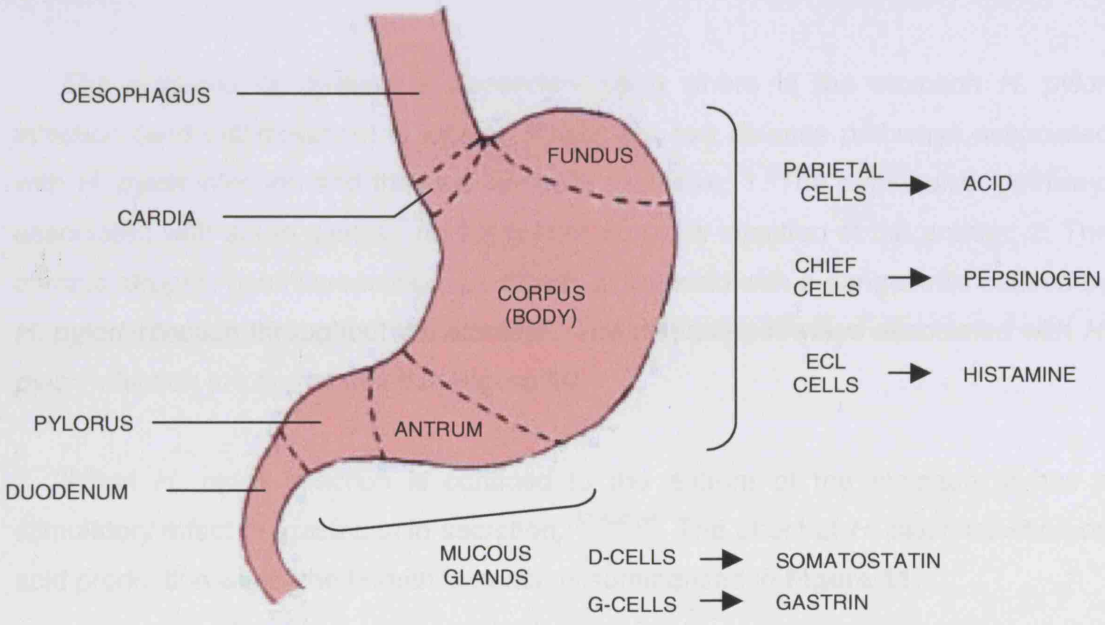

Figure 9: Diagram showing regions within the human stomach and their respective cell types (adapted from a diagram in the 'Gastric histology' lecture of Dr. Driman, Dept. of Pathology, LHSC University, USA.).

In the normal stomach, eating stimulates the parietal cells (via the vagus nerve) to secrete acid in the body of the stomach. Food entering the stomach stimulates the G cells (located in the antrum) to release gastrin, a hormone that binds to and stimulates parietal cells in the body to secrete acid.[551] Gastrin also binds to ECL cell receptors and stimulates them to release histamine, which acts as a regulator of acid secretion.[552] Histamine in turn binds to H2 receptors on parietal cells leading to complete acid release. Further acid secretion is inhibited by feedback pathways which operate as the acidity of the stomach and duodenum increase.[552] For example, D cells in the antrum release the hormone somatostatin, which inhibits gastrin release from the G cells by paracrine control.[553]

H. pylori specifically infects the human stomach (tissue tropism), where it preferentially and primarily colonises the antral region.[554,555] It is not known entirely why *H. pylori* has preference for the antrum of the stomach but it is thought to be the result of a number of factors including acid regulation (higher acid content in the corpus limits *H. pylori* growth) and possibly differential expression of receptors within the antrum and other stomach regions.[556-559] Very little work has been done investigating the adhesin-receptor interactions involved in adhesion of *H. pylori* to different regions of the stomach. These factors will be discussed in more detail in Chapter 7.

The outcome of disease is dependent upon where in the stomach *H. pylori* infection (and inflammation) is located. There are two disease pathways associated with *H. pylori* infection and they are mutually exclusive: 1. The peptic ulcer pathway, associated with acute gastritis resulting from *H. pylori* infection of the antrum; 2. The chronic atrophic gastritis-carcinoma pathway associated with a pangastritis caused by *H. pylori* infection throughout the stomach. The disease pathways associated with *H. pylori* infection are summarised in **Figure 10**.

When *H. pylori* infection is confined to the antrum of the stomach, it has a stimulatory effect on gastric acid secretion.[557,560,561] The effect of *H. pylori* infection on acid production within the human stomach is summarised in **Figure 11**.

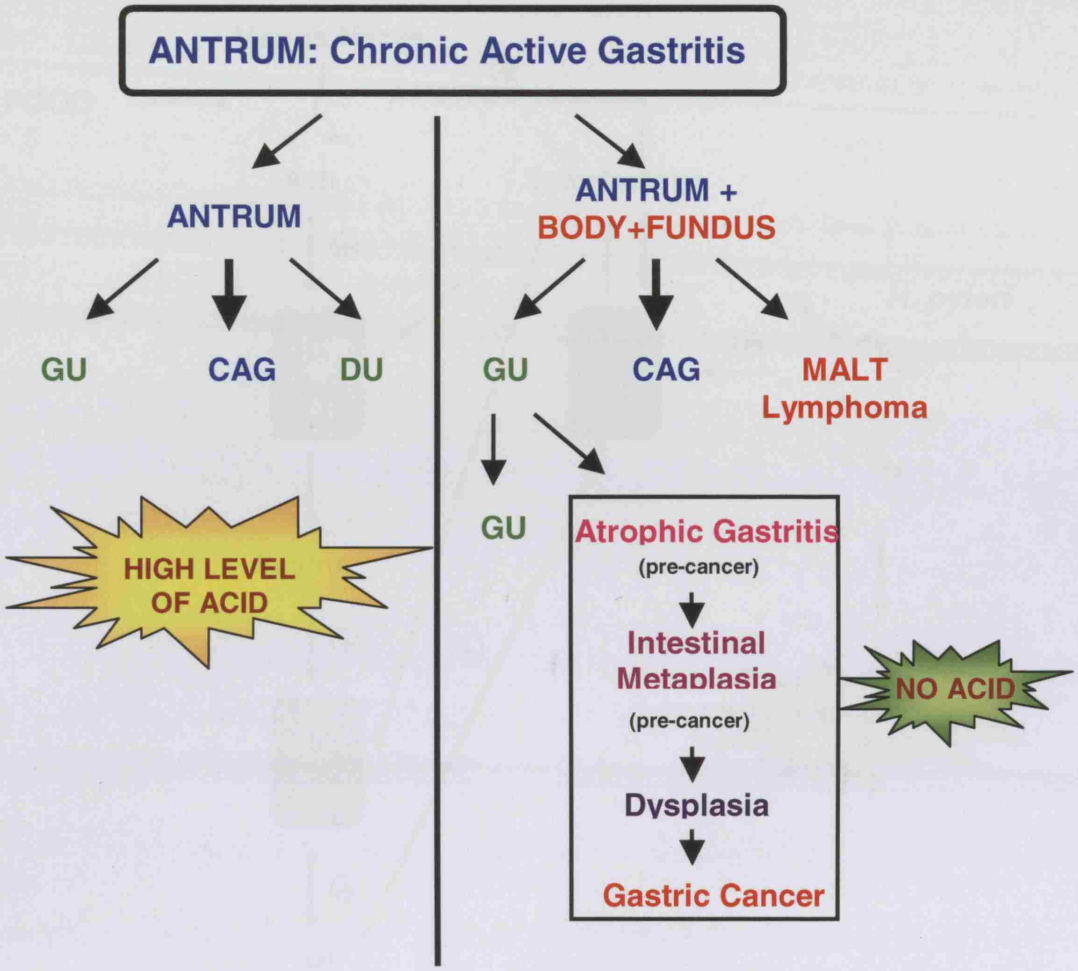

Figure 10: The two disease pathways associated with *H. pylori* infection.

H pylori gastritis causes a reduction in somatostatin levels and since somatostatin negatively regulates gastrin, this results in hypergastrinemia and therefore excess acid.[562-565] The release of gastrin is further enhanced by a positive feedback loop: because gastrin is a specific growth factor for *H pylori*,[566] inflammation is increased (due to increase numbers of *H. pylori* cells) and thereby so is the release of gastrin.[506] *H. pylori* induced proinflammatory cytokines may also directly stimulate G cells leading to enhanced expression of gastrin.[567,568] Because enterochromaffin-like (ECL) cells (also known as oxyntic cells, found near the basement membrane of the stomach mucosa) and parietal cells are mostly absent in the antrum, the higher levels of gastrin lead to increased acid secretion by the body mucosa where these cells are located.[569]

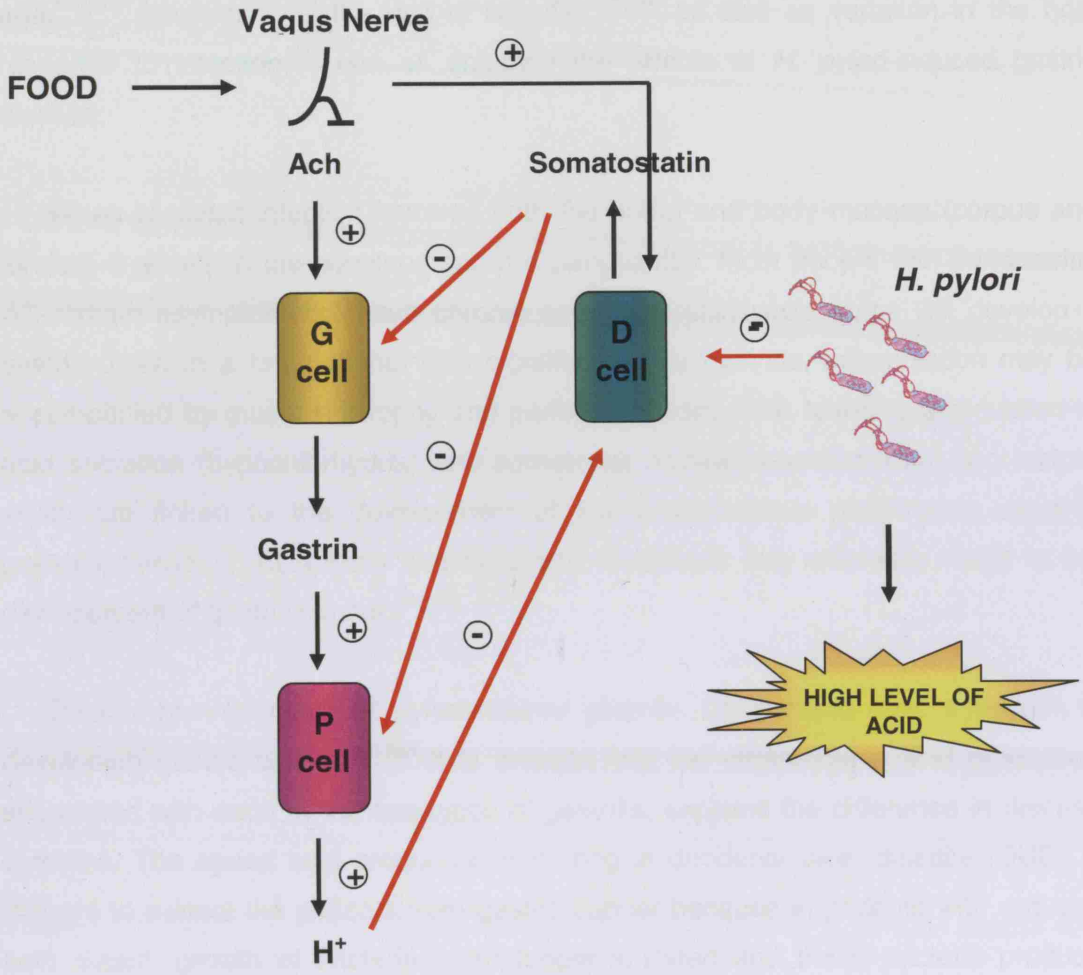

Figure 11: The effect of *H. pylori* infection on acid production within the human stomach.

These effects are enhanced because increases of gastrin lead to increases in parietal cell mass.[569,570] The increased release of the hormone gastrin results in acid hypersecretion. This increase in acid leads to a heightened acid load on the duodenum.[560] *H. pylori* also has the effect of reducing bicarbonate secretion in the duodenum thereby lowering the tissue's resistance to the increased levels of acid.[571,572] Together, these effects result in damage to the duodenal mucosa and induce the formation of gastric metaplasia within the duodenal bulb. Since *H. pylori* can colonise gastric-type cells, *H. pylori* infection is now able to spread to the duodenum where it causes further damage.[573,574] Mucosal integrity is breached resulting in the development of inflammation, erosive duodenitis and ulceration. Acid

however, is not the only factor involved in duodenal ulcer formation: stress; [575] age;[576,577] smoking;[578,579] the use of NSAIDs;[576,580] as well as variation in the host response to infection,[581] can all enhance the effects of *H. pylori*-induced gastric disease.

When *H. pylori* infection involves both the antral and body mucosa (corpus and fundus) it results in the development of a pangastritis. Most people with pangastritis will remain asymptomatic (have chronic active gastritis) while some will develop a gastric ulcer. In a few patients with significant body gastritis, inflammation may be accompanied by mucosal atrophy and parietal cell loss. This leads to a reduction in acid secretion (hypochlorhydria) and sometimes complete achlorhydria; two factors which are linked to the development of the precancerous phenotypes atrophic gastritis, intestinal metaplasia and dysplasia. Dysplasia may ultimately result in the development of gastric cancer.[551,582]

Despite having severe *H. pylori*-induced gastritis, DU patients have a low risk of developing gastric cancer.[551,583] It is thought that the difference in acid production associated with each of the two types of gastritis, explains the difference in disease outcome. The raised acid production occurring in duodenal ulcer disease (DUD) is thought to protect the patients from gastric cancer because in patients with reduced acid output, growth of bacteria is no longer inhibited and these bacteria produce potentially carcinogenic nitrosoamines.[584-586] There is also a dysregulation in cell turnover, which leads to increased apoptosis and in response to this, an increase in cell replication; a high risk factor for the development of cancer.[587,588] The findings of many studies support the association between *H. pylori* induced hypochlorhydria and the increased risk of gastric cancer.[589-592] This may be the effect of the host having a specific IL-1β polymorphism which enhances the production of IL-1β, a pro-inflammatory cytokine that also inhibits gastric acid secretion.[590]

Genetic variations such as a person's acid secretory status and parietal cell mass will also affect the amount of acid produced by the infected person and whether infection with *H. pylori* leads to either the duodenal ulcer or the gastric cancer pathway. Hypersecretors (as for patients with antral infection) are most susceptible to duodenal ulcer (and protected against pangastritis/gastric cancer) because of the increased acid production.[551] Hyposecretors on the other hand (as for patients with pangastritis/corpus-predominant infection), do not produce enough acid and thus infection from the antrum is able to spread to the body. *H. pylori* infection has also been shown to increase the amount of nitrites and decrease the amount of Vitamin C

in gastric juice,[593-595] (which leads to the production of carcinogenic N-nitroso compounds), and this, in patients with hypochlorhydria or achlorhydria who have a diet high in nitrates or nitrites and low in Vitamin C, makes them more susceptible to the development of gastric cancer.[596,597]

In addition to the aforementioned disease pathways, *H. pylori* infection can also lead to the development of MALT (mucosal associated lymphoid tissue) Lymphoma. This consists of cancerous growths of immune cells that are recruited to secretory tissues such as the gastrointestinal tract.[598] Inflammation of the stomach lining induced by *H. pylori* infection can result in the development of MALT-type lymphoid tissue. Once the MALT-type tissue has developed, lymphocytes are continuously stimulated (as a result of the constant presence of large numbers of bacteria; a normal immune response) to replicate and increase in number.[599] In a small minority of people, however this results in a genetic mutation of a lymphoid cell (rendering it resistant to apoptosis) and the continual proliferation of this faulty cell line leads to the development of a lymphoma.[600] Additionally, it has been shown that *H. pylori* activates NF-KappaB in B lymphocytes resulting in anti-apoptosis which may also result in carcinogenesis.[601]

1.4.3 Influence of *H. pylori* virulence factors on disease outcome

Virulence genes are one factor which has been shown to affect the outcome of disease in infected patients. *H. pylori* strains can possess a variable number and combination of different virulence genes. VacA has several allelic variants, the VacA *s1a/m1 genotype has* been linked to gastric cancer and peptic ulcer disease and the *s1b/m2* genotype linked to MALT lymphoma.[517,602] CagA is thought to be involved in gastric cancer and peptic ulcer development.[514,603] IceA (induced by contact with gastric epithelial cells) is thought to be linked to peptic ulcer disease[518,520] and oipA (outer inflammatory protein A) is thought to be involved in the development of both peptic ulcer disease and gastric cancer.[603]

BabA2 is the gene encoding the blood-group antigen-binding adhesin BabA which is involved in adherence of *H. pylori* to the Lewis b blood group antigens on gastric epithelial cells. The findings of Gerhard et al.[604] suggest that in German adults the *babA2* gene is associated with a higher risk of *H. pylori* related diseases and that the gene has a strong association with both *cagA* (80% *babA2* positive) and *vacAs1* genotypes (79% *babA2* positive).

It has been shown that patients infected with strains of *H. pylori* that co-express certain virulence genes, are at the highest risk of developing the more serious diseases. In particular, according to the studies of Koehler et al.[517] patients with strains harbouring the vacA s1a and iceA1 genotype are at a higher risk of developing gastric cancer. The studies of Zambon et al. [605] who looked at other virulence genes, concluded that patients infected with strains co-expressing cagA, vacA s1/m1 alleles and babA2, had the worst degrees of inflammation and intestinal metaplasia, a pre-cancerous condition. It has also been proposed that the development of ulcer or adenocarcinomas is related to the presence of the triple positive genotype *vacAs1 cagA* and *babA2*.[604] Others suggest that the *vacA s1b m1, cagA, cagE, iceA2* genotype lead to more severe *H. pylori*-induced gastrointestinal infections.[485,518,606,607] Apart from *H. pylori* virulence genes, many and other factors are involved in *H. pylori*-induced diseases. These include acid production, host and environmental factors. These are discussed further in Chapter 7.

1.4.4 Adhesion

Even though *H. pylori* possesses and requires a number of different virulence factors for colonisation, survival and pathogenesis in the human stomach, one of the most essential factors for colonisation and the pre-requisite for the initiation of disease, is adhesion to the gastric epithelium. Specific adhesion of *H. pylori* to the gastric mucosa involves a number of different adhesins that are associated with the attachment of the bacterium to three main types of structure: gastric mucin, components of the epithelial cell ECM or the epithelial cells themselves. Once *H. pylori* has reached the gastric mucosa it either binds to the mucin or degrades the mucin allowing it to swim through and bind to the underlying epithelial cells or the ECM. The adhesin-receptor interactions identified to date for *H. pylori* are summarised in **Table 3.**

1.4.4.1 Adhesion to the gastric extracellular matrix

Despite being a non-invasive pathogen it is interesting that *H. pylori* utilises ECM proteins for adherence. Logan et al.[608] suggest that ECM components may be important secondary binding sites; adhesion occurring when *H. pylori* interferes with the intercellular junction or when the epithelial cells of the stomach are shed. It is also thought that ECM adhesion may be important in damaged tissue when the basement membrane of the host cells becomes exposed.[609]

1.4.4.2 Adhesion to gastric mucin

Although *H. pylori* can adhere to components of the gastric mucus, it is rarely found deep in the gastric mucosa. This is because mucin from this part of the mucosa is a natural antibiotic. It contains O-glycans which inhibit *H. pylori* cell wall synthesis.[610]

TFFs

Environmental conditions of the stomach do not fully explain the tropism of *H. pylori* for the human stomach since areas of gastric metaplasia in the duodenum allow *H. pylori* to colonise this region, suggesting that there is a specific *H. pylori* receptor or receptors present in gastric tissue.[611] So far no specific gastric *H. pylori* receptors have been identified which explain this tropism. For example, Lewis b and Lewis x are also expressed in the duodenum and *H pylori* has been shown *in vitro* to bind to duodenal tissue expressing Le b.[612]

Fairly recently a new group of proteins called trefoil factors have been identified.[613] These peptides are specifically found in the mucus granules secreted by mucus-secreting cells. They are involved in the process of restoration after mucosal injury and protect the integrity of the epithelial barrier.[153,614] The expression of TFFs in the epithelium of the GI tract is site specific. TFF1 is found in the surface foveolar epithelial cells of the stomach,[615] TFF2 in the deep glands of the distal stomach but not in the pits or surface mucosa[616] and TFF3 is expressed by goblet cells in the large and small intestine.[617]

Gene expression of mucin glycoproteins and trefoil peptides has been shown to be correlated.[152] In the body and superficial antral region of the stomach TFF1 is associated with MUC5AC, in the fundus and deeper antral glands TFF2 and MUC6 are coexpressed and throughout the mucosa of the large and small intestine TFF3 and MUC2 are found together.[618] MUC5AC but not MUC6 has been shown to be associated with *H. pylori* and is not found in intestinal metaplasia unless they are of an incomplete phenotype which express MUC5AC.[619] Clyne et al.[611] have recently shown that this association can be explained by the fact that *H. pylori* interacts with TFF1 which may therefore act as an *H. pylori* receptor *in vivo*. Although the Le b receptor has been found in MUC5AC,[620] *H pylori* can bind to MUC5AC when Le b is absent .[612] In the study of Clyne et al.[611] nearly all strains bound to TFF1 but not to Le b; these two observations therefore let the authors to suggest that interaction with TFF1 may explain the tropism *H. pylori* has for gastric tissue. Other studies suggest the involvement of Lewis b binding to MUC5AC,[620] however these studies have used

purified mucin which is likely to have removed TFF1.[611] The exact epitopes responsible for *H. pylori* binding to TFF1 have not yet been identified.

1.4.4.3 Adhesion to gastric epithelial cells

Multiple adhesin-receptor interactions have been described for *H. pylori* adhesion to gastric mucosa. Both fucosylated glycoproteins and sialylated glycolipids have been shown to be important *H. pylori* binding sites in the gastric epithelium.[15,53,621] Of those described, the best studied adhesin-receptor interaction for *H. pylori* is between the blood-group antigen binding adhesin (BabA) [622] found on the outer surface of the bacterium and the Lewis b (Le b) blood group antigen[15,57] which is expressed in bodily secretions, on erythrocytes and by gastric, duodenal and some distal colonic epithelial cells.[623-626]

Lewis (Le) blood group antigens are characterised into two main groups based on the type of carbohydrate backbone they possess: Type 1 antigens (Le a and Le b) have a Type 1 carbohydrate backbone and Type 2 antigens (Le x and Le y) are derived from Type 2 carbohydrates.[532] Synthesis of Lewis antigens is controlled by two fucosyl transferase (*FUT*) genes on chromosome 19. *FUT3* codes for the Lewis enzymes and controls the synthesis of Lewis antigens. The second gene *FUT2* controls the expression of the antigens on cell surfaces (e.g. mucosal epithelial cells) and in bodily secretions (e.g. saliva and colostrum). *FUT1* encodes the H1 enzyme responsible for producing the glycans that are converted into the Lewis antigen.[627] Persons who express and secrete the ABH, Le b and Le y antigens are known as 'secretor' individuals and those who express and secrete Le a and Le x are known as 'non-secretors'.[628] The structure and synthesis of blood group antigens are summarised in **Figures 12 and 13.**

People of ABO blood group type O have been shown to have more *H. pylori* Le b receptors[15] which is probably why blood group O persons have a higher incidence of *H pylori* infection and hence are at a higher risk of developing *H. pylori* related diseases such as peptic ulcer. [631,632,633]

Gastric sialylation itself is induced by *H. pylori* infection. It is thought that the chronicity of *H. pylori* infection and virulence of the organism is contributed to by the ability of many strains to bind to such sialylated glycoconjugates expressed during chronic inflammation.[621] Mahdavi et al.[621] also suggest that *H. pylori* adhesion to sialylated glycoconjugates contributes to the increased risk of PUD development in

infected non-secretor individuals (people lacking the ABO blood group antigens in secretions such as saliva and milk). This is because these individuals lack the H1 and Le b antigens, and in their place they have sialyl Le x (sLe x) and sialyl Le a (sLe a) antigens. *H. pylori* strains that express the SabA adhesin will thus be able to adhere better than strains expressing only BabA, and thus the potential of these strains for causing PUD is increased in such individuals.

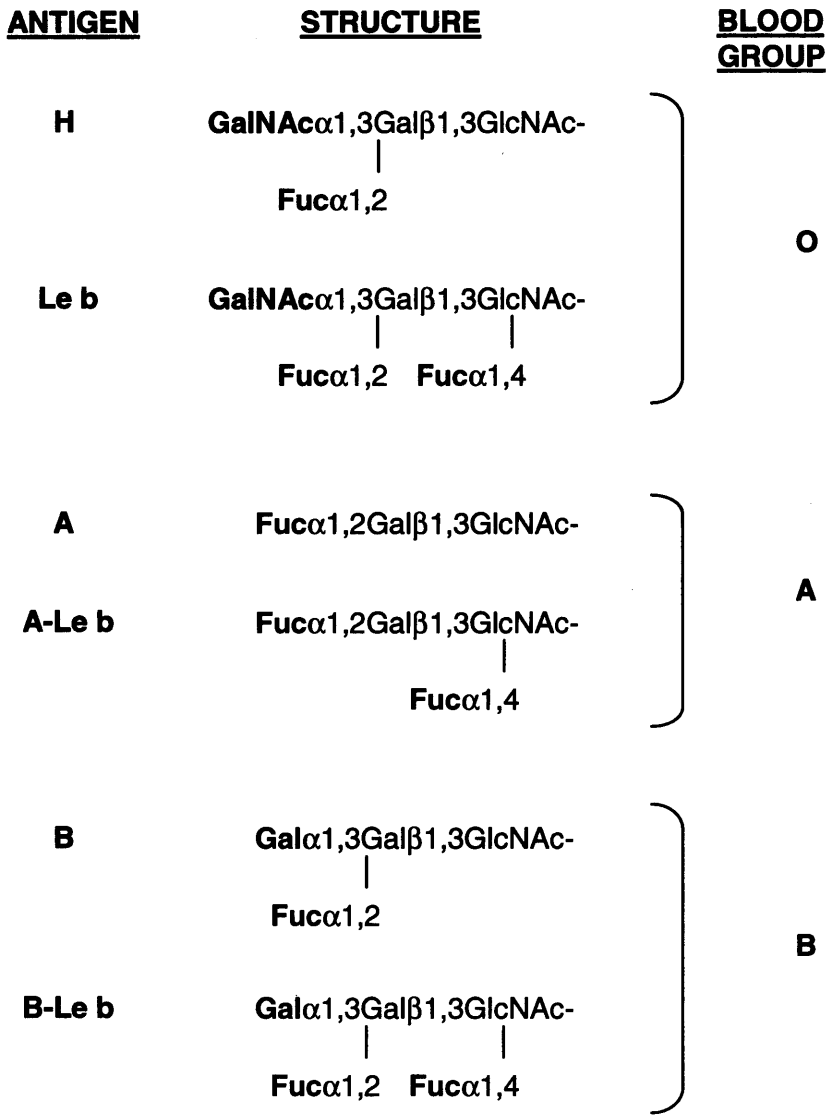

Figure 12: Structures of fucosylated blood group antigens and their relationship to the ABO system. Fuc = fucose, Gal = Galactose, GalNAc = N-acetylgalactosamine (after Aspholm-Hurtig et al.)[634]

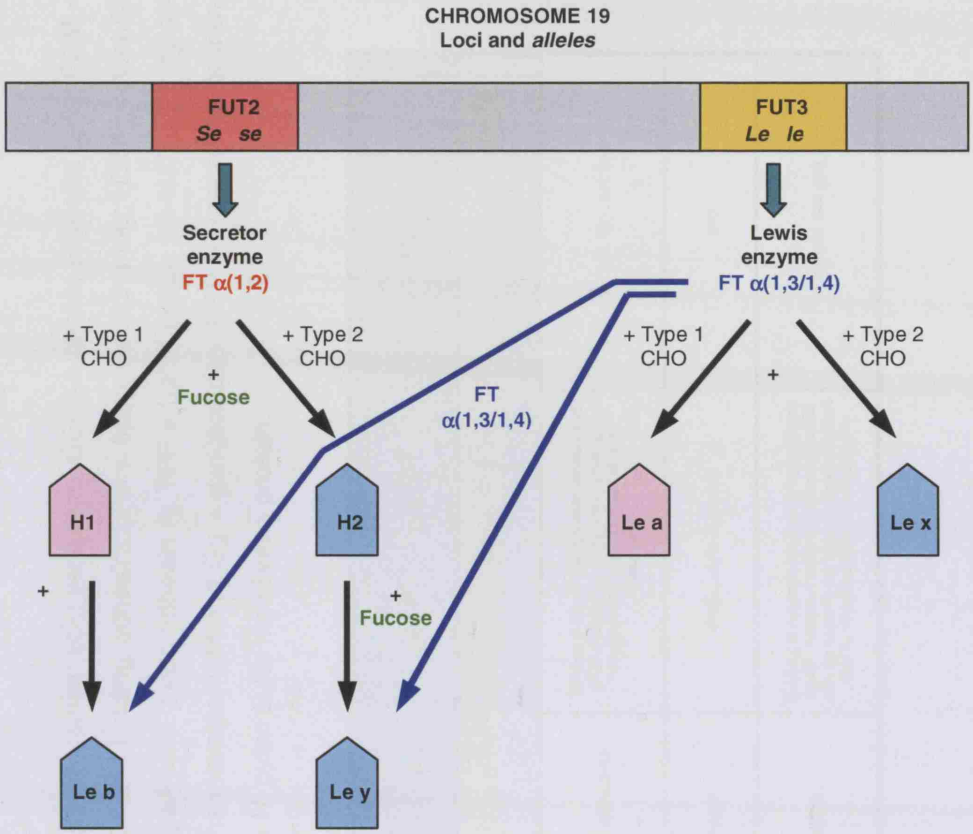

Figure 13: Synthesis of blood group antigens by the fucosyl transferase genes (FUT) of chromosome 19. *Se* and *se* = secretor alleles, *Le* and *le* = Lewis alleles. CHO = carbohydrate.

Summary

Broadly speaking, adhesion has been described as a two-step process.[621] The BabA-Lewis b interaction (BabA is the major *H. pylori* adhesin) occurs when the bacterium is further away from the epithelial surface. As it draws nearer, a second interaction comes into play between the SabA adhesin of *H. pylori* and its receptor sLe x on epithelial cells, which protrudes less from the epithelial surface than Le b (Le b is found on surface glycoproteins whereas sLe x is found in membrane glycolipids). During inflammation *H. pylori* triggers the host tissue to modify gastric carbohydrate patterns to up-regulate sLe x, the antigen associated with inflammation. Then SabA binds to this antigen and thus *H. pylori* attaches even closer to the membrane where its ability to gain nutrients from the host cells is improved. However, the interactions of multiple *H. pylori* adhesins are likely to be involved in the adhesion of the bacterium to the gastric mucosa and these are summarised in **Table 3**.

Table 3: Adhesin-receptor interactions involved in *H. pylori* adhesion.

Hp = *H. pylori*; CHO = carbohydrate; GAGs = glycosaminoglycans; RGD = Arg-Gly-Asp amino acid sequence; GECs = gastric epithelial cells; ECM = extracellular matrix; BabA = Blood group antigen binding adhesin; SabA = sialic acid binding adhesin; LPS = lipopolysaccharide; HSBP = heparin sulfate binding protein; NLBH = Neuraminyl-Lactose-Binding-Haemagglutinin; HpaA = *H. pylori* adhesin A; TFF = trefoil factor; Hp-NAP = *H. pylori*-neurophil activating protein; PGC = Polyglycosylceramide; PE = phosphatidylethanolamine; GM and Gg = gangliosides; HSP = Heat shock protein; Alp = Adherence-associated lipoprotein; Oip = Outer inflammatory protein; Hop = *H. pylori* outer membrane protein.

ADHESIN	ADHESIN EPITOPE	RECEPTOR	RECEPTOR EPITOPE	TYPE OF INTERACTION	TEST METHOD / TISSUE	REFERENCES
Specific adhesion						
Extracellular matrix						
SabA (66-kDa protein)	Unknown	Laminin	Carbohydrates containing epitopes α2-3-linked sialic acids (eg. α2-3-linked *N*-acetyl neuraminic acid); terminal sialic acids	Lectin (Protein-CHO)	Binding of Hp to radiolabelled laminin and ECM proteins; Overlay assay binding to neoglycoconjugates on nitrocellulose membranes	53, 609, 621, 641-643
LPS	Phosphorylated structure in core oligosaccharide	Laminin	Unknown	Unknown	Binding of Hp to laminin	642
HSBP (possibly 42.7-kDa or 55-60-kDa) protein)	Unknown	Heparin and heparan sulphate proteoglycans; sulfatides, GAGs	Sulphate not CHO component of heparin	Unknown	Binding of heparan sulphate to immobilised HSBP; Inhibition of Hp adhesion to gastric cells by heparin; Hp binding to radiolabelled GAGs, heparin and heparan sulphate	15, 609, 644-649

Table 3 continued

ADHESIN	ADHESIN EPITOPE	RECEPTOR	RECEPTOR EPITOPE	TYPE OF INTERACTION	TEST METHOD / TISSUE	REFERENCES
Extracellular matrix (continued)						
Possibly NLBH (HpaA lipoprotein) adhesin or 25-kDa lectin; sialic acid specific haemagglutinins	Possibly HpaA subunit protein of NLBH (has sialic acid-binding motif)	Vitronectin	Sialic acid moieties	Lectin (Protein-CHO)	Binding of Hp to radiolabelled vitronectin	14, 609, 650
57-kDa and 42-kDa proteins	Unknown	Plasminogen	Within the 5th Kringle structure (fold of the protein)	Protein-protein	Binding of Hp to radiolabelled plasminogen	14, 609, 651
Protein (not BabA or SabA)	Unknown	Fibronectin	Integrin-like (RGD motif)	Unknown	Overlay assay binding to neoglycoconjugates on nitrocellulose membranes; Binding of Hp to radiolabelled fibronectin	53, 609, 639, 642
Unknown	Unknown	Collagen (Type IV)	Unknown	Unknown	Binding of Hp to radiolabelled collagen	14, 609, 641
Unknown	Unknown	Fibrinogen	Unknown	Unknown	Binding of Hp to radiolabelled fibrinogen	14
Gastric Mucin						
unknown	Unknown	Trefoil Factor 1 (TFF1; protein)	Unknown	Unknown	Binding of Hp to TFF1 assessed by Flow Cytometry and BIAcore; inhibition of Hp adhesion by TFF1 to porcine mucin	611
Gastric Epithelial cells						
BabA (78-kDa protein)	Unknown	Le b and H1 blood group antigens	L-fucose	Lectin (Protein-CHO)	Tissue sections (normal human stomach); Overlay assay binding of Hp to neoglycoconjugates and blood group antigens; Adhesion of Hp to radiolabelled blood group antigens	15, 53, 57,622
SabA (66-kDa protein)	Unknown	sLe x, sLe a	Carbohydrates containing epitopes α2-3-linked sialic acids	Lectin (Protein-CHO)	Overlay assay binding to neoglycoconjugates on nitrocellulose membranes; Binding of Hp to tissue sections and immobilised antigens on TLC plates; Hp binding to sialic acid and inhibition of haemagglutination	53, 621, 641, 652

Table 3 continued

ADHESIN	ADHESIN EPITOPE	RECEPTOR	RECEPTOR EPITOPE	TYPE OF INTERACTION	TEST METHOD / TISSUE	REFERENCES
Gastric Epithelial cells (continued)						
LPS	Le x structure in O-antigen side-chains	Unknown	Unknown	Unknown	Binding of Hp mutants and Le x coated beads to human tissue sections	529, 530, 537, 637, 639, 653, 654
Hp-NAP (150,000-kDa protein)	Unknown	Sulfatide, gangliotetraosyl ceramide, sulfated mucins and blood group antigens (sulfo-3-Le a, sulfo-3-Le x and Le x)	Unknown	Unknown	Binding of Hp components to salivary mucin and oligosacchraides by ELISA; Hp binding to glycosphingolipids TLC immunostaining; Inhibition of Hp adhesion to gastric cell monolayers by CHOs, glycoproteins and glycolipids; Hp adhesion to sulfatide-coated plates	528, 655-658
NLBH (32-kDa protein)	HpaA (lipoprotein) subunit contains a sialic acid-binding motif	3-linked and α-2,3-linked sialic acids (e.g. 3'SL), *N*-acetyl-neuraminyl(α-2,3)-lactose	Unknown	Unknown	Binding of adhesin to fetuin; Hp haemagglutination of erythrocytes carrying sialic acids	652, 659-665
PGC (polyglycosylceramide)-binding adhesin (non-NLBH)	Unknown	Polyglycosylceramides	Unknown	Unknown	Binding of Hp to PGCs and other sialated glycoconjugates on TLC plates	635, 636, 666
PE/lipid-binding adhesin (M-selectin); 63-kDa protein	catalase??	Lipids PE and lyso-PE, GM_3 Gg_3 and Gg_4	Unknown	Unknown	Adhesion of Hp to PE, GM_3 Gg_3 and Gg_4 by TLC overlay Adhesion to phospholipid receptors immobilised on TLC plates	51, 667-669
HSP60 and HSP70 (60 and 70-kDa proteins)	Unknown	sulfatides; lactoferrin and other glycoproteins and glycolipids with similar CHO structures	Unknown	Unknown	Inhibition of Hp adhesion by anti-HSP60 mAbs to gastric carcinoma cells, primary human epithelial cells; binding of Hp to immobilised receptors	670-675
AlpA Lipoprotein	Unknown	Unknown	Unknown	Unknown	Adhesion of Hp mutants to human gastric tissue sections and colonisation of guinea-pig *in vivo*	39, 676, 677

Table 3 continued

ADHESIN	ADHESIN EPITOPE	RECEPTOR	RECEPTOR EPITOPE	TYPE OF INTERACTION	TEST METHOD / TISSUE	REFERENCES
Gastric Epithelial cells (continued)						
AlpB Lipoprotein	Unknown	Unknown	Unknown	Unknown	Adhesion of Hp mutants to human gastric tissue sections and colonisation of guinea-pig stomach *in vivo*	39, 677
OipA	Unknown	Unknown	Unknown	Unknown	Colonisation of Hp mutants *in vivo*mouse stomach; deJonge et al., (2004) say not involved in adhesion to guinea-pig stomach.	676, 678
HopZ protein	Unknown	Unknown	Unknown	Unknown	Adhesion of Hp mutants to gastric epithelial cells and tissue sections and colonisation of mouse stomach *in vivo*. deJonge et al., (2004) say not involved in adhesion to guinea-pig stomach.	676, 679-681
HP1188 and HP1430 proteins	Unknown	Unknown	Unknown	Unknown	Adhesion of GECs to nickel beads coated with the adhesins,	683

1.4.5 Consequences of *H. pylori* adhesion

As mentioned previously, *H. pylori* adhesion results in reduced gastric mucin synthesis, increased gastric acid production and leads to the development of morphological changes in host cells (adhesion pedestals and hummingbird phenotypes). However, colonisation also induces a strong immune response; an attempt by the host to clear the infection.

1.4.5.1 Host Immune responses

Host cells only initiate an inflammatory response to *H. pylori* once it has adhered to the gastric epithelium and it is enhanced when the bacteria inject the cytotoxins CagA and VacA.[684-686] The immune response to *H. pylori* infection comprises two major components: the innate response and adaptive response.

Innate immune response

Colonisation of *H. pylori* results in the recruitment of inflammatory cells to the gastric mucosal surface. Contact of the bacterium with the epithelial surface or the release of virulence factors such as CagA or OipA to the epithelium, triggers the release of IL-8 by epithelial cells which attracts lymphocytes and monocytes to the immediate vicinity.[686-688] Activation of these inflammatory cells is supported by the presence of *H. pylori* LPS and urease.[689-691] Lymphocytes produce cytokines that direct the immune response towards the Th-1 (inflammatory) pathway.[692,683] However, the severity of the inflammatory response depends upon host genetic factors such as IL-1 genotype (which determines the level of pro-inflammatory cytokine expression) and of bacterial virulence factors such as cagPAI, LPS and VacA.[467,603] LPS is known to influence the type of immune response to *H. pylori* infection depending upon the presence of LPS Lewis antigens. Expression of these antigens is phase-variable and can be switched on and off by the bacterium. If present, the Lewis antigens bind to lectins (DC-SIGN) on DCs which blocks Th1 development. Strains which are LPS-Lewis negative do not bind to DCs and therefore induce a strong Th1 cell response.[694] The Th1 response may be appropriate for *H. pylori* infection because it is associated with inflammation and gastritis; the host attempting to clear persistent infection. In mice and human tissue the Th1 response has been shown to reduce *H. pylori* colonisation whereas a Th2 response favours protection.[695,696]

The Th1 response may be an adaptation of the host to attempt to remove *H. pylori* infection or an adaptation of *H. pylori* to maintain persistent infection, since components of the Th1 response have both beneficial and deleterious effects on *H. pylori*.[696-698] Studies show that *H. pylori* seems to preferentially trigger IL-12 secretion by DCs (a Th1 cytokine) and this response may be important in controlling infection as it appears to reduce colonisation.[696] A Th2 response is also initiated (IL-10 release) which may reduce inflammation and the cytotoxic effects of Th1 cells.[698] The Th1 response (release of IFNγ) results in gastritis (increased gastrin and reduced levels of somatostatin),[699] whereas IL-4 production (a Th2 response) has been shown to reduce gastrin secretion and colonisation of *H. pylori*; which may be why *H. pylori* suppresses IL-4 production. This suggests that *H. pylori* may drive a Th1 response in order to encourage persistent infection.[697,700] Additionally, the mechanisms whereby *H. pylori* suppresses the immune system (especially via the action of VacA; see section 1.4.5.2) could allow persistant colonisation and therefore gastric inflammation (Th1 response) because it impedes an effective Th2 and hence also an adaptive immune response.

Antigen-presenting cells (APCs) such as monocytes and dendritic cells (DCs), recognise bacterial molecules (e.g. LPS) via pattern-recognition molecules such as toll-like receptors (TLRs), in particular TLR2, 4 and 5[701-703] resulting in the activation of these immune cells which respond by secreting pro-inflammatory cytokines (such as TNFα, IL-1β and IL-8). The studies of Rad et al.[704] have shown that *H. pylori* infection is associated with increased amounts of these cytokines. These cytokines act as local chemoattractants, inducing infiltration of granulocytes (neutrophils/PMN).[705,706] The monocytes differentiate into macrophages, which phagocytose bacteria and destroy them by intracellular lysis. *H. pylori* however, by interfering with lysosomal proteins and the action of catalase breaking down H_2O_2, is able to survive intracellularly within the macrophage.[707] As mentioned previously, phagocytosis of *H.* pylori by epithelial cells has been shown to occur *in vitro*, inside of which the bacteria are able to survive; however this phagocytosis is partly induced by bacterial factors probably to avoid the host immune system.[708,709] One of the major *H. pylori* antigens recognised by the human immune system is urease; it helps recruit monocytes and neutrophils to inflamed mucosa and activates the production of inflammatory cytokines.[710,711]

Adaptive immune response

Because *H. pylori* infection is persistent at the gastric mucosal surface, a specific immune response can be mounted by the host towards characteristic bacterial

proteins. *H. pylori* sheds antigenic material and LPS, disrupts epithelial integrity and produces toxins; all of which have the potential to induce such a response.[712,713] The gastric mucosa produces both IgA and IgG against *H. pylori*.[714-718] IgA antibodies bind to *H. pylori* and block adhesion to the gastric mucosa or are involved in their opsonisation.[57, 715,719]

H. pylori is a strong activator of DCs.[720] Bacterial proteins are phagocytosed by DCs and together with MHC and co-stimulatory molecules, are expressed on their surface. Antigen-presentation by the DCs leads to $CD4^+$ T-cell activation, these cells react to the antigens by producing cytokines that stimulate B cells to produce antibodies and cause cell lysis.[4]

H. pylori infection induces strong antibody responses in the human gastric mucosa; B cell numbers and B cell production of specific antibodies against *H. pylori* antigens are increased.[717,721-723] *H. pylori* antigens which stimulate antibody production include HSP60 (which induces IL-6 produced by macrophages and epithelial cells and stimulates immunoglobulin secretion), flagellin and urease (which result in the production of *H. pylori*-specific IgM antibodies.[724,725]

1.4.5.2 *H. pylori* mechanisms of immune evasion

Despite such a florid/substantial immune response to infection, *H. pylori* has developed an array of mechanisms to down-regulate and avoid host immune defences which are activated when it adheres to the gastric epithelium. These include:

1. **Interference with phagocytosis** - Phagocytosis by macrophages is delayed by virulent (Type I) strains of *H. pylori* which have been shown to persist inside novel vacuoles (megasomes). Even though Type I strains strongly activate PMN they are able to disturb targeting of NADPH oxidase so that the reactive oxygen species produced by this enzyme are diverted away from the phagosome and instead accumulate in the epithelial cell environment.[726-728] Superoxide dismutase and catalase enzymes of *H. pylori* also protect the bacterium from damage by free radicals within phagocytes: these enzymes convert the radicals into oxygen and water.[729]
2. **Minimising Innate immunity** – *H. pylori* LPS has a low toxicity, that is although it stimulates macrophages via TLR4 it does not stimulate the TLR4 on gastric epithelial cells.[730] The DNA of most bacteria is largely unmethylated and this is recognised by TLR9 which stimulates an inflammatory response.[731]

Because *H. pylori* has highly methylated DNA[732,733] it is therefore able to avoid recognition by the host immune system through this route .

3. **Downregulation of immune effectors** – VacA of *H. pylori* has several effects on immune effectors. It interferes with the uptake and processing of antigens, which is important for antigen-presentation in the acquired immune response, suppresses activation and proliferation of T-cells and in addition the cytotoxin CagA can induce T-cell apoptosis.[498,499,734-736] *H. pylori* also impairs memory T-cell responses and therefore could aid persistence of infection.[737]
4. **Mimicry of host antigens** – *H. pylori* is able to evade recognition by the adaptive immune system because it expresses the host antigens Le x and Le y on the O chain of its LPS, which serve as camouflage from the immune systen due to molecular mimicry.[533]
5. **Antigenic variation** – Another way *H. pylori* evades adaptive immune recognition is by varying the expression of certain surface proteins;[738] this high-frequency antigenic variation is mediated by gene mutations.[739,740]

1.5 Treatments and antibiotic resistance

Microbial infection involving bacteria or fungi are most commonly treated by giving patients a course of broad-spectrum antibiotics. The main drawbacks of using antibiotics are that they often kill the normal members of the microflora, may have harmful side-effects on the patient and always eventually lead to the development of drug resistance by the target microorganisms. There are currently a number of different antibiotics available to treat *C. albicans* and *H. pylori* infection.

1.5.1 Treatments

1.5.1.1 *C. albicans* infection of the vagina

Currently, the main class of antimycotics used to treat VVC are the azoles. The type of treatment given for *C. albicans* infection of the vagina depends upon whether the infection is considered 'complicated' or 'uncomplicated'.[741] The guidelines for treatment have been set out by the Centres for Disease Control (CDC) and can be found at their website.[951] Complicated VVC is diagnosed if one or more of the following applies to the patient: infection is recurrent; infection is severe; infection is

with *Candida* species other than C. *albicans*; the woman has uncontrolled diabetes mellitus, immunosuppression / debilitation or is pregnant. Uncomplicated VVC is diagnosed if any of the following criteria apply: infection is infrequent or sporadic, inflammation is mild to moderate; the main cause of infection is *C. albicans*; the woman does not have immunosuppression.

If infection is classed as complicated, then the recommended treatment[951] consists of topical application of antimycotic because this has been shown to be more effective than a single oral dose, but treatment should be extended to 10-14 days duration.[742,743] If oral trearment is preferred by the patient for severe VVC, then two doses of fluconazole should be taken 3 days apart, which has been shown to be more effective than a single oral dose.[744] Additionally, if the patient has severe discomfort as well as the infection, a low-potency steroid cream and topically applied fungal cream may be favourable.[745]

If infection is uncomplicated, then there are several standard treatments available, these are listed in **Table 4**. All standard treatments have been shown to be equally effective and have a cure rate of approximately 80%.[743]

AGENT	DOSAGE
Butoconazole 2% vaginal cream	5g per day intravaginally for 3 days
Butoconazole 2% vaginal cream (sustained release)	5g intravaginally, one application
Clotrimazole 1% vaginal cream	5g per day for 7 to 14 days
Clotrimazole 100mg vaginal tablet	One tablet per day intravaginally for 7 days or two tablets per day intravaginally for 3 days
Clotrimazole 500mg vaginal tablet	One tablet intravaginally, one applictaion
Fluconazole	One 150mg tablet, taken once orally
Miconazole 2% vaginal cream	5g intravaginally for 7 days
Miconazole 100mg vaginal suppository	One suppository per day intravaginally for 7 days
Miconazole 200mg vaginal suppository	One suppository per day intravaginally for 3 days
Nystatin 100,000 Untit vaginal tablet	One tablet per day intravaginally for 14 days
Tioconazole 6.5% ointment	5g intravaginally, one application
Tioconazole 0.4% vaginal cream	5g per day intravaginally, for 7 days
Tioconazole 0.8% vaginal cream	5g per day intravaginally, for 3 days
Tioconazole 80mg vaginal suppository	One suppository per day intravaginally for 3 days

Table 4: Standard treatments for uncomplicated VVC (after Owen and Clenney;[741] based on CDC guidelines).

For recurrent VVC (RVVC), the recommended treatment is a long-term maintenance therapy once initial therapy has been completed.[746,951] RVVC is diagnosed if a patient has had four or more incidences of yeast infection in one year and this is not due to reversible causes (for example, antibiotic therapy or the contraceptive pill). The cause of RVVC is usually attributable to a person having diabetes mellitus or immunodeficiency, or if the infection is dominated by non-albicans species, which are more resistant to azole therapy.[746] However, *C. albicans* resistance to azoles is increasing. Resistance of *C. albicans* (from the oral and vaginal mucosa) to azoles such as fluconazole seems to be very high, since prevalence of *C. albicans* is very high in HIV patients most of whom have had considerable azole exposure.[724]

The two recommended regimens for RVVC (either topical intavaginal or oral application) and maintenance therapies are outlined in **Table 5**.

AGENT	DOSAGE
Clotrimazole	One 100mg tablet once a day intravaginally for 7 days
Clotrimazole (for maintenance)	One 500mg tablet intravaginally once a week
Fluconazole	One 150mg tablet taken once orally and again after 3 days
Fluconazole (for maintenance)	One 500mg tablet orally once a week

Table 5: Standard and maintenance treatments for RVVC (after Owen and Clenney;[741] based on CDC guidelines).

1.5.1.2 *H. pylori* infection

Treatment of *H. pylori*-induced gastroduodenal diseases (i.e. symptomatic *H. pylori* infection) has developed over the years from a laborious, one month-long therapy, to a 1-week triple therapy and a 'treatment package' agreed upon internationally, has lead to the formulation of the Maastrict 2 guidelines in the year 2000.[747,748] This report states that the first-line of eradication therapy for *H. pylori* should be a 7-day triple therapy using a proton-pump inhibitor (PPI) or rantidine bismuth citrate (RBC) combined with two antibiotics, clarithromycin and amoxycillin or metronidazole to

replace amoxicillin in patients allergic to penicillin. If the initial regimen fails then a second-line therapy should be used. This should be a 7-day quadruple therapy consisting of a PPI, colloidal bismuth subcitrate, metronidazole and tetracycline. This two-step treatment package avoids the development of dual antibiotic resistance since clarithromycin and metronidazole are not given together as a first-line therapy. Overall eradication rates of the package have been shown to be between 95-99%.[749-751]

The purpose of the PPI is to block the production of stomach acid so that it does not interfere with the activity of the agent, or accentuate any damage already present in the patient's stomach. PPIs are substituted benzimidazoles which are weak bases and lipophilic. They are thus able to cross the parietal cell membrane and once inside the parietal cell, where the environment is acidic, they became protonated and produce the activated form of the drug, sulphenamide. The active form binds covalently to the enzyme that operates the gut's proton pump (H^+/K^+ ATPase), resulting in irreversible inhibition of acid secretion by the proton pump.[752-754] Examples are omeprazole, lansoprazole, rabeprazole and pantoprazole.

H2 receptor antagonists (H2RAs) are alternative agents to PPIs for stopping acid secretion in the stomach.[755] These agents reversibly inhibit the action of histamine on the histamine H2 receptor found on gastric parietal cells. Gastrin stimulates ECL cells in the stomach body/corpus (which have gastrin receptors) to produce histamine. These bind to the histamine receptors found on neighbouring parietal cells which as a result are stimulated to produce acid. By blocking the histamine receptors, stomach acid secretion is inhibited.[755] Examples are Ranitidine, Cimetidine, Famotidine and Nizatidine.

Bismuth or bismuth compounds were originally included as part of the treatment packages because they were thought to coat the stomach or ulcer and protect it from acid.[756] It is now known that the added benefit of adding bismuth to a treatment regimen is due to its antibacterial effect on *H. pylori*,[757,758] however on its own it is unable to completely eradicate *H. pylori*.[759-761] Examples are rantidine bismuth citrate (RBC) bismuth compound combined with the H2RA ranitidine.

Because treatment contains a combination of an antisecretory agent and antibiotics, several studies have looked at the effectiveness of the antisecretory agent used. All have concluded that the efficacy of PPI and H2 receptor antagonist are similar and the efficacy of various PPIs are also very similar.[762,763]

Due to the development of *H. pylori* resistance to the currently available antibiotics, new lines of therapy often involving new antibiotics, are constantly being sought after and developed. Several groups have tested third-line therapies, to be used if the first and second-line treatments fail. For example, a two-week quadruple therapy of PPI (omeprazole), tetracycline, metronidazole and bismuth subcitrate was given to patients after the failure of two previous treatments and resulted in an eradication rate of 95%.[764] A 7-day regimen of PPI (omeprazole), rifabutin and amoxycillin in patients previously exposed to clarithromycin, metronidazole and tetracycline, gave an eradication success of 79%[765] and a study using the same therapy with low and high dose rifabutin showed that the high dose was more effective, eradication rates were 66.6% and 86.6% respectively.[766] However, results using rifabutin in clinical practice were very disappointing and one study only achieved a 38% eradication rate.[767] Combination therapies for first, second and third-line treatments involving lefloxacin have been much more successful[768-780] and when treatment was extended from 7 to 10 days with lefloxacin, a PPI and amoxycilin, it resulted in 83.3% eradication.[771]

New 1st-line regimens have been summarised in two recent reviews[747,772] and involve new agents such as lansoprazole, tinidazole, pantoprazole, amoxycillin-clavulanic acid, rabeprazole and esomeprazole as part of triple or quadruple therapies. Several new promising agents include clavulanic acid (a β-Lactamase inhibitor) and furazolidone (a nitrofuran which inhibits RNA production). Trials using clavulanic acid have shown it to be effective against *H. pylori in vivo*. When given in combination with amoxycillin, clarithromycin and omeprazole, clavulanic acid gave an 86.6% eradication rate compared with 66.6% eradication for the same treatment without clavulanic acid.[773] Furazolidone a new possible antibiotic, produced an 88.9% success rate in patients when given in combination with a PPI and amoxycillin, however using this higher dose caused some negative side-effects.[774] Two other new antibiotics, azithromycin and rabeprazole have also been shown to be effective as part of triple therapies in recent human trials.[775,776] NE-2—1 has been shown to be a good antimicrobial agent *in vitro* against *H. pylori*[777] and may therefore be an agent used in future trials. Araujo et al.[778] used tetracycline, furazolidone and colloidal bismuth citrate which gave an 88.2% eradication rate for a 4 day therapy.

Interestingly, it has been recently shown that using more prolonged therapy than the traditional 7 days, (for example 10 or 14 days) leads to a higher eradication rate of *H. pylori* infection. Additionally, the use of sequential therapy for 10 days (i.e. 5 days of a PPI plus amoxicillin followed by 5 days of triple therapy with a PPI, tinidazole and

clarithromycin) has been shown to enhance eradication rates compared with normal triple therapy.[779-783]

Due to the lack of sensitivity and cost of antimicrobial susceptibility testing, routine testing of patients' *H. pylori* strains is not recommended to determine the most effective antibiotics against *H. pylori* for use in combination therapy. However, in areas of high antibiotic usage where antibiotics used in standard regimens are likely to be ineffective, sensitivity testing prior to treatment may be beneficial.[772]

1.5.2 Antibiotic resistance

Because microorganisms have a very short generation time they are able to produce rapid responses to environmental changes. When the selective pressure of an antimicrobial agent is introduced into their environment, microbes respond by becoming resistant, that is they remain capable of reproducing and surviving even in the presence of the agent.

Studies that have tried to determine the prevalence of microbial resistance to antibiotics have several major limitations because they often involve too few patients or recruited patients are not representative of patients as a whole. Also, there are many different methods used to detect resistance, some of which are known to be less accurate; no gold standard has been introduced. Additionally, because data gathering for such studies is a lengthy process and publication is often delayed, the majority of available data for prevalence of resistance comes from the end of the last decade. This is useful mainly for showing trends but does not give an accurate indication of the current status.

1.5.2.1 Mechanisms of resistance

Acquired resistance is a consequence of changes in the cellular structure and physiology of a microorganism and is brought about by modifications in its normal genetic makeup. Most modifications are caused by genetic mutations, by the transfer of genes from one organism to another enabling the acquisition of resistance genes or by a combination of both.

There are three main mechanisms by which microorganisms may develop resistance to the effects of an agent: [784]

1. **Prevention of intracellular drug accumulation by:**
    i) introducing changes into the outer microbial membrane so that the drug is no longer able to bind to the surface of the microorganism
    ii) terminating active transport of the drug across the cell membrane
    iii) upregulating active efflux mechanims to pump the drug out of the cell before it damages the microorganism
2. **Modification of the part of the microbe that the drug targets**, resulting in ineffective levels of drug binding to the target site.
3. **Production of a drug-inactivating enzyme** that reduces or even eliminates the ability of the drug to kill the microorganism.

1.5.2.2 *C. albicans* resistance to antifungals

Antifungals are easily imported through the lipid bilayer of fungi by passive diffusion, owing to the drugs being hydrophobic in nature.[785] There are five major classes of antifungals, classified according to their mode of action.

1. **Azoles** – these are the most common class in use and inhibit the synthesis of ergosterol (a major component of fungal cell membranes) by blocking the enzyme responsible 14α-lanosterol demethylase, coded for by the *ERG11* gene. This results in disruption to the structure of the fungal cell membrane. Point mutations in the *ERG11* gene lead to changes in the affinity of azoles to their target protein and also leads to resistance.[786] Examples are the triazole antifungals Fluconazole, Voriconazole and Itraconazole.
2. **Allylamin** – this agent also block ergosterol synthesis (via gene *ERG1*), resulting in a build-up of toxic squalene.[787]
3. **Polyenes** – bind directly to ergosterol in the fungal cell membrane forming pores that make the cell membrane less permeable (leaky) resulting in cell death. An example is Amphotericin B.[788]
4. **Candins** – inhibit the synthesis of the major fungal cell wall polymer β-1,3-glucan and thus interfere with cell wall synthesis An example is the echinocandidin Caspofungin.[789]
5. **Pyramidines** – inhibit the synthesis of nucleic acids by inhibition of thymidylate synthase.[790,791] An example is the nucleotide analogue Flucytosine, which inhibits RNA and DNA synthesis in fungal cells that take up the drug.

Resistance in *Candida* is multifactorial; different mechanisms may appear as the organism adapts during the course of antifungal treatment.[792,793] The main mechanisms employed by *C. albicans* that confer resistance are:

1. **Alterations in the biosynthetic pathway of ergosterol.** This is brought about by overexpression of the *ERG11* gene which codes for the target enzyme of the drug, 14α-lanosterol demethylase.[791,794]
2. **Modifications of the target enzymes by point mutations** thereby reducing the affinity of the enzyme for the drug.[795-799]
3. **Reducing the intracellular accumulation of the agent.** This is brought about by overexpression of genes encoding drug-efflux pumps. *Candida* cells which show an increase in the expression of genes encoding drug-extrusion pumps also show simultaneous increase in the efflux of drugs. This prevents *Candida* cells accumulating lethal concentrations of azoles and enables survival.[800,801]

In addition to sterols, phospholipids of the fungal membrane have also been shown to be altered by antifungal agents.[802,803] Interestingly, inactivation of drugs (metabolic conversion), a common resistance mechanism seen in bacteria, has not been observed in *Candida*. Also unlike bacteria, fungi cannot pass on resistance genes from strain to strain because they do not contain a plasmid or have a known mechanism capable of transferring genetic material between strains. Each isolate within a patient infected with *C. albicans*, seems to develop resistance by responding locally to the selective pressure of drug exposure. Because direct transfer of genes does not occur in fungi, the development of resistance in these microorganisms is not as fast as in bacteria. The ability of *C. albicans* to develop an antifungal resistance genotype is not a cost-free adaptation. Trade-offs occur in fitness, resulting in the reduced reproductive output of the microorganism.[804,805]

1.5.2.3 Resistance in *H. pylori*

All of the known resistance mechanisms used by *H. pylori* for the development of antibiotic resistance involve point mutations. Because point mutations arise on the chromosome, the spread of resistance is limited since they can only be transferred to the descendant organisms (vertical transfer). Horizontal transfer is thought to be possible by transformation and genetic exchanges between different strains of *H. pylori* seem to be numerous. Transfer by transformation has been shown for metronidazole *in vitro,*[806] however this type of transfer does not seem to occur in most cases.

The three main types of antibiotics most often used to treat *H. pylori* infection are a macrolide (clarithromycin), a 5-nitroimidazole (metronidazole) and a β-Lactam.[807]

1. **Macrolides** – these target *H. pylori* 23S rRNA, in particular domain V to which they bind, and therefore cause disruption of protein synthesis.[808,809] *H. pylori* responds by producing point mutations so that the macrolide is no longer able to bind to the ribosome.[810] Generally a high prevalence of resistance has been reported for clarithromycin; resistance rates vary depeding upon usage and in some places where use has been limited, the prevalence is still fairly low.[811,812]

2. **Nitroimidazoles** – In order to be active, 5-nitroimidazoles such as metronidazole must first be reduced inside the bacterial cells. Reduction leads to the release of cytotoxic compounds that damage subcellular structures and cause mutations in the DNA which are lethal to the bacterium.[813] The redox potential of the bacterial cell must be lower than the drug in order to reduce it. Redox systems in anaerobes are capable of doing this but not those of aerobes. *H. pylori* is usally able to reduce metronidazole as it is a microaerobe.[814] Mutation of the *rdxA* gene (encoding oxygen-insensitive nitroreductase), thereby inactivating it, is a major resistance response of *H. pylori* to 5-nitroimidazoles.[815] Enzymes such as nitroreductase which maintain the redox potential of the bacterial cell, function as electron donors which reduce the drug and activate it. In addition to nitroreductase, there are several other enzymes within *H. pylori* that have been shown to reduce metronidazole.[816-820] Prevalence of resistance to metronidazole is quite high in many countries, but not in Japan since use of the drug is infrequent.[811,812]

3. **β-Lactams** – β-Lactams operate by binding to regulatory enzymes such as penicillin-binding proteins (PBPs) which catalyse the cross-linking of the main structural component of the bacterial cell wall, peptidoglycan. In growing bacteria this results in inhibition of cell wall synthesis and leads to cell death. The only β-Lactam currently used to treat *H. pylori* is amoxycillin. Resistance to this drug is seldom found at the moment but is expected to increase in the future when the outcome of selective pressure will be seen.[811,812] Dore et al.[821,822] have shown that many strains of *H. pylori* lack PBP which is thought to be why they are able to tolerate the drug. In other bacteria, resistance develops by modifying the PBP or by the production of β-Lactamase, which inactivates the antibiotic and this is also thought to be true of *H. pylori*.[821,823]

Additional antibiotics used for *H. pylori* treatment are:

β-Lactamase inhibitors – these have been designed in response to the resistance mechanism of *H. pylori* to β-Lactam antibiotics. Because *H. pylori* produces β-Lactamase enzyme to inactivate the antibiotic, by introducing an agent which inhibits the action of β-Lactamase, the antibiotic is able to have its desired effect upon the bacteria.[824] In a recent trial, Ojetti et al.[773] have shown that addition of the β-Lactamase inhibitor clavulanic acid to a triple therapy greatly increases the success rate of eradication.

Fluoroquinolones - these bind to the *gyrA* gene encoding DNA gyrase, the enzyme involved in relaxing the supercoiling of DNA to allow its replication. Resistance is induced by point mutations in the *gyrA* gene.[825] Quinolones such as cerfloxacin have a good efficacy since resistance rate is low except in countries such as Portugal which have a high consumption rate of such drugs.[811,812]

Rifacmycins – The target of these drugs is the β-subunit of RNA polymerase. The main drug in use is rifabutin. No resistance has been reported *in vivo* so far[811,812] however *in vitro*, point mutations in the gene coding this subunit have been shown to confer resistance.[826]

Tetracyclines – Tetracyclines (for example tetracycline) target and bind to the 16S rRNA gene, thus interfering with protein synthesis. Mutation in a region adjacent to the tetracycline-binding site of this gene has been shown to result in resistance of *H. pylori* to this drug.[827,828] Resistance to tetracycline currently seems to be rare, except in Korea.[811,812]

1.5.2.4 Consequences of antibiotic resistance

In addition to the development of resistance by pathogens, using antibiotics to treat microbial infection also has adverse effects on the resident microflora. Antibiotics usually kill all non-pathogenic organisms present which may allow room for pathogenic organisms to colonise if the microflora is not rapidly replaced, for example by taking probiotic supplements alongside antibiotic treatment.[811] Another disadvantage of antibiotic therapy is lack of patient compliance due to adverse events, strong antibiotics may have harmful side-effects on patients which lead to early termination of therapy. One study showed lack of compliance in over 10% of patients.[829] With the rapid development of antibiotic resistance in microorganisms, adverse effects on host and microflora, a need exists for the development of novel, alternative strategies to treat microbial infection.

1.6 Novel treatments for Infection

Although the use of molecular methods can rapidly detect antibiotic resistance and host polymorphisms, which may lead to reduced efficacy of treatment and thus eradication failure,[812,830] this is not a long term solution to the rising trend of antibiotic resistance. However many new antibiotics are discovered which have static effects or can kill microorganisms, it is almost certain that these microbes will develop resistance to all the agents developed. Additionally, the speed at which new agents are made available for use (discovered and tested for safety and efficacy) is unlikely to be able to keep up with the rate at which these organisms develop resistance to those in current use, in order to provide replacements. It is therefore necessary to look for new sources of antimicrobial agents as well as new strategies to successfully combat microbial infection, which circumvent the mechanisms (namely interference with growth, metabolism or killing), which induce microorganisms to develop resistance. New sources of antimicrobials and new strategies to fight infection are discussed below.

1.6.1 Discovery of new sources of antimicrobials

a) ***Plants and plant products.*** Investigations based on ethnobotanical studies have found that in many cases, plants can provide a natural source of antimicrobial substances which have cidal, static and anti-adhesive effects on many different microorganisms.[831,832]

b) ***Animal products.*** Colostrum and milk from humans and animals have been found to be a source of many different types of antmicrobial molecules including antibodies, lactoferrin and carbohydrates which may act as anti-adhesins.[833-835] Products such as propolis or honey both produced by bees have been shown to have antimicrobial properties against *H. pylori* and *C. albicans.*[836-838]

c) ***Food products.*** Melanoidins which form in heat-treated foods have been shown to have antibacterial properties against *H. pylori.*[839] Glycopeptides in buttermilk have been found to have anti-adhesive properties on microorganisms such as *H. pylori.*[840]

d) ***Other.*** Mushrooms, marine microorganisms, gill tissue of mussels, earthworms and genes from flatfish have also been shown to provide novel sources of antimicrobial agents.[841-845]

1.6.2. Development of agents to overcome microbial resistance mechanisms

Inhibitiors of efflux pumps. Because microbial efflux pumps are not very specific for substrates (i.e. they work against many different antibiotics), agents which inhibit or modulate the proteins of efflux pumps could block pump activity, thus increasing the effectiveness of antibiotic treatment. Efflux protein inhibitors have been shown to increase the susceptibility of *Candida* to azoles.[846-850]

1.6.3. Specific targeting of antimicrobial drugs

a) ***Probiotics.*** Members of the normal microflora can be genetically modified to secrete antimicrobial agents (peptides or antibodies) against pathogenic bacteria. Because the commensals bind to the target tissue they therefore target the therapy to the exact site of infection. For example, Medaglini et al.[851] used *Streptococcus gordonii* to deliver yeast killer cytotoxins in the vagina and were shown to be as effective as fluconazole at killing *C. albicans* in a rat model of VVC. Midolo et al.[852] and Coconnier et al.[853] showed that *L. acidophilus* strains inhibit the growth of *H. pylori* not through lactic acid production but probably by secreting bacteriocin-like proteins. The presence of microflora may also simply 'displace' attached pathogenic microorganisms. Lactobacilli against *C. albicans* in the vagina have been shown to do both: they produce antifungal peptides and displace *C. albicans.*[854]

b) ***Photodynamic therapy.*** A non-toxic dye (photosensitiser) is targeted to a destination cell or microbe by combining the agent to a molecule that binds to a specific molecule displayed on the surface of the target cell, or one that passes through the target cell membrane. When low intensity visible light is applied to the area of infection, in the presence of oxygen cytotoxic products are produced killing either the infected cell or the organism.[855]

c) ***Bioadhesives.*** Bioadhesives such as microbeads, nanoparticles or mucoadhesives can be complexed to antimicrobials thus enabling them to bind to and directly deliver the drug to the mucosal surface and to prolong the delivery of drugs to the site of infection. This strategy has been shown to be effective against *H. pylori* (reviewed by Conway et al.)[856-858] Mucoadhesive gels containing clotrimazole have been shown to be effective against *C. albicans* infection of the vagina in rats.[859]

d) ***Mucolytic agents.*** By removing or disrupting the surface mucus layer, mucosal delivery of antibiotics can be improved. This has been shown in humans using pronase and a combination of antibiotics as treatment against *H. pylori* infection of the stomach.[860]

e) ***Prolonging drug residence at the site of infection.*** A number of investigations (reviewed by Conway et al.[856] have attempted to prolong the residence of drugs against *H. pylori* in the stomach by using systems which float on the gastric contents (to avoid being rapidly removed with the exit of fluid), ion-exchange resins (drug-resin complex is broken down at the site of infection) and swelling and expandable systems (which expand in the stomach and become too large to exit through the pyloric sphincter).

1.6.4. Immune enhancement and vaccine development

Many previous studies have shown that effective protective immunity against both *C. albicans* and *H. pylori* infection can be induced in experimental animals after immunisation with appropriate antigens (for example urease, CagA, Hp-NAP, MPs, Saps or whole attenuated cells) and adjuvants (eg. aluminium hydroxide or Freund's adjuvant). Recent data have shown for *H. pylori* that the most effective manner of inducing a strong local and systemic immune response was to prime animals mucosally (orally or intranasally).[861] Because contraction of *H. pylori* infection occurs during childhood, prophylactic vaccination would need to be given to young babies. Ideally the development of a therapeutic vaccine would be more appropriate especially in western countries where *H. pylori* infection is only treated when patients become symptomatic.[862] Several investigators have designed methods whereby protection against microbial infection can be induced by drinking milk: cows are immunised with microbial antigens so that antibodies against them are produced in the milk.[863]

For *C. albicans* infection of the vagina, the evidence generally suggests that local immunity rather than systemic (humoral and/or cellular), plays a defensive role[433,864-867] and vaccinations which lead to protective immune responses have been obtained by local immunisation.[864,865,868.869] It has been shown in rats that both intravaginal and intranasal immunisation (using immunogenic antigens such as *C. albicans* MP extract or Saps) induce the production of vaginal antibodies and confer protection against vaginal candidiasis.[864,865,868-870]

1.6.5 Inhibition of adhesion

However, most of these strategies still involve the use of antimicrobial agents with static or cidal effects, which would yet again eventually lead to the development of resistance in the target microorganisms. An alternative strategy, developed to overcome the problems of resistance, is to target the first step of microbial infection: to inhibit adhesion of the organism to the host tissue.[3] Because inhibition of adhesion works on the principle of sterically blocking microbes from attaching to host tissue, rather than a microbicidal effect, the selection of inhibitor-resistant strains is unlikely to occur.[871] If the microorganism can be prevented from binding, then it is less likely to invade and establish infection, resulting in tissue damage and host debilitation. Inhibitors may also be able to 'remove' organisms that are already bound, thus providing a therapeutic as well as prophylactic application.

As described earlier (section 1.1.2.3), microbes adhere to tissue by specific ligand-receptor interactions. Therefore, by using molecules (analogues) that mimic the microbial adhesin or its complementary host cell receptor, or by using antibodies that target the adhesin or receptor, it may be possible to block microbial adhesion to the host tissue (**Figure 14**).

The concept of inhibiting microbial adhesion is now receiving more serious attention by many researchers and increasing awareness of its potential use as an alternative therapy to antibiotic-based treatment has occurred particularly over the last 5 years. A number of studies have shown both *in vitro* and *in vivo* the effectiveness and plausibility of such a treatment for human microbial infections, however, only a handful of studies have been carried out in human clinical trials (see **Table 11**, Chapter 5).

One of the most up and coming as well as promising sources of anti-adhesives in recent years has been from plants. Both the extracts and products of certain plants have been shown to inhibit microbial adhesion both *in vitro* and *in vivo.* There have been many different methods used to investigate inhibitors of microbial adhesion, however, one major drawback of most has been the lack of a quick and accurate method of quantification. These topics will be addressed further in Chapters 3 and 6.

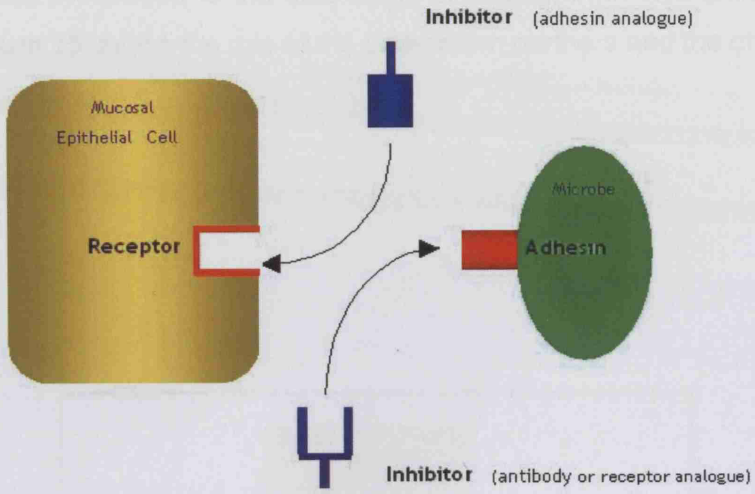

Figure 14: Principles of inhibition of microbial adhesion. Block the first step of infection, the adhesion of the microbe to the host cell, by designing inhibitors (analogues) that mimic the adhesin or receptor.

In 2001, an EU-funded consortium known as 'ADRI' was set up, (under the EU Quality of Life and Management of Living Resources Programme, Framework 6: Control of Infectious Diseases). The main objective of this consortium was to develop novel therapies for *H. pylori*, *C. albicans* and rotavirus infection, based on the principle of inhibition of adhesion (see http://www.ucl.ac.uk/adri/). Part of the work involved in this thesis formed an integral component of the ADRI project. Some of the partners were involved in producing the inhibitors (domain antibodies, minibodies or glycoconjugates) while others were involved in the screening and testing of the inhibitors. This is the stage in which I was involved. I received potential inhibitors of both *H. pylori* and *C. albicans* adhesion from the other partners, which had already been tested at the molecular level for their binding affinity using surface plasmon

resonance (Biacore machine) in order to initially screen them. The best candidates from the molecular screening were then sent to me for testing in the *in vitro* binding assay system that I set up (see Chapters 2 and 3). Agents found to inhibit adhesion in my system then proceeded to the next stage of testing, in *in vivo* animal models of infection. **Figure 15** shows the role of the consortium partners and the different stages of the project.

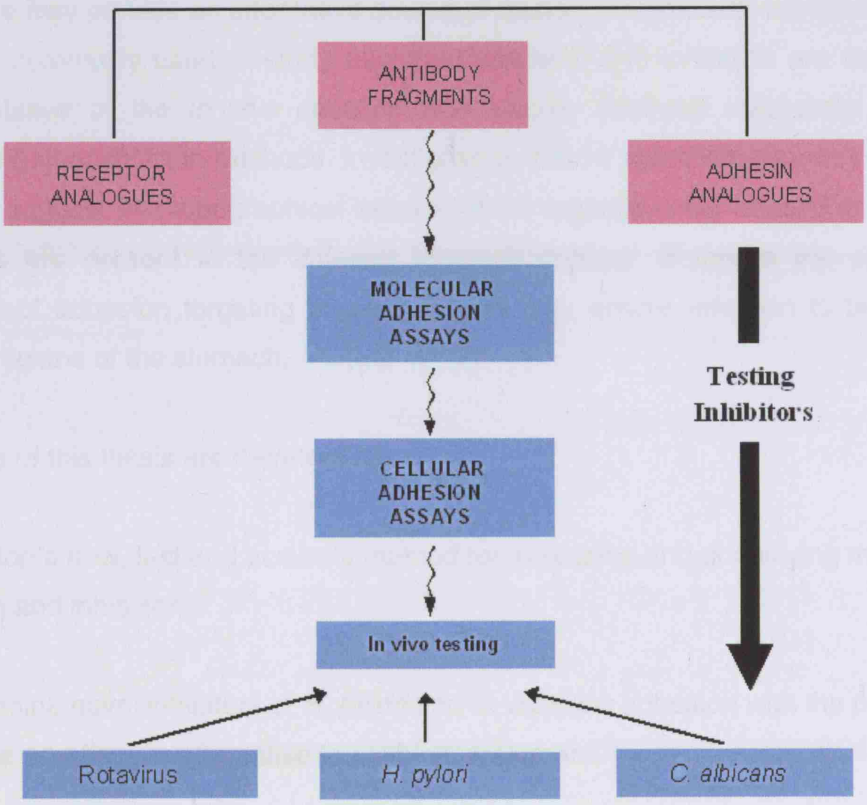

Figure 15: Diagram showing the role of the ADRI consortium partners at the different stages of the project.

1.7 Aims and Objectives of Thesis

Some superficial mucosal infections may become chronic leading to a persistent acute inflammatory reaction and in some cases persistent symptoms for the patient. In the case of infection with *Helicobacter pylori* and *Candida* vaginitis, the host is unable to eradicate the organism from the mucosa. In both cases the effectiveness of antimicrobial agents is being compromised by an increasing level of resistance of both *Helicobacter* and *Candida* to the appropriate agents. These two microorganisms are therefore prime candidates for investigations into new therapies such as inhibition of adhesion. Several plants have been shown to have antimicrobial effects on *H. pylori* and these may provide an alternative source of bactericidal and anti-adhesive agents. Methods commonly used to study microbial adhesion and inhibition are usually not representative of the *in vivo* situation and involve relatively inaccurate or time-consuming quantification methods. Investigations into *H. pylori* infection of the human stomach suggest that topographical location of the organism may depend upon which receptors are present in the different stomach regions. If this is the case then inhibitors of adhesion targeting these receptors may enable infection to be cleared from all regions of the stomach.

The aims of this thesis are therefore to:

1. Develop a new, fast and accurate method for assessing and quantifying microbial adhesion and inhibition.

2. Determine novel inhibitors of *H. pylori* and *C. albicans* adhesion with the potential to provide an effective alternative to antibiotic treatment

3. Investigate the antimicrobial properties of a number of culinary and medicinal plants against *H. pylori.*

4. Investigate the adhesin-receptor interactions involved in adhesion of *H. pylori* to different topographical regions of the human stomach.

SECTION II

METHODS

Chapter 2

MATERIALS AND METHODS

(Optimisation of Adhesion/Inhibition Assay)

To assess adhesion and inhibition of adhesion of *C. albicans* and *H. pylori* to the mucosal epithelium, an *in vitro* assay using tissue sections was used, based on the assay developed by Boren et al.[15] and Falk et al.[57] whereby binding of the organisms to tissue sections is observed by fluorescent (confocal) microscopy. The assay consists of four main parts. The first part deals with the organisms themselves: they are grown, counted and a defined number are fluorescently labelled. The second part involves the tissue: small pieces of tissue are processed (frozen or fixed and paraffin-embedded) and sectioned. Thirdly, the labelled organisms and tissue sections are incubated together in an adherence (or inhibition) assay, the tissue is also fluorescently stained and the sections are viewed with a scanning laser confocal microscope. The fourth step is quantification: digital photos of the sections are taken from the microscope and with the aid of image analysis software, the number of organisms adhering to the epithelial surface of the tissues are counted.

Optimisation of the assay conditions was an important pre-requisite for further work on assessing the inhibitors and was a major part of this thesis. Optimisation included organism and tissue preparation as well as the development of the quantification method (Chapter 3). Because this method was the basis for all subsequent studies of the thesis, steps taken for optimisation are outlined below.

All reagents were purchased from Sigma-Aldrich, UK unless otherwise stated.

2.1 Microbial isolates

H. pylori NCTC 11637 and *C. albicans* ATCC 90025 were used in the study.

2.1.1 Growth conditions

2.1.1.1 *H. pylori*

H. pylori is usually grown for 2-5 days on 5% horse blood agar with Columbia agar base.[872] The CCUG (Culture Collection University of Göteborg, Sweden) states that *H. pylori* has a varying growth time). Experiments were performed to assess the effect of prolonged growth on binding.

It is generally thought that under stressful conditions (such as antibiotic treatment, acidic conditions, aerobic culture, alkaline pH and long-term incubation), *H. pylori* transforms from spiral form to its coccoid form.[853-857] Although both the spiral and coccoid forms have been observed in the human stomach,[878-881] in epithelial cells of biopsies[882] and in culture,[873] the viability and ability to adhere of the coccoid form is very controversial.[873,883,884] In the studies of Osaki et al.[885] adhesion of the coccoid form to MKN45 human gastric carcinoma cells was significantly lower than that of the helical form.

Methods: *H. pylori* NCTC 11637 was grown for a variable time up to 5 days on Columbia blood agar (Oxoid, UK) at 37°C under microaerophilic conditions using CampyPak (Oxoid, UK).[872] After every 24hrs a few of the colonies were removed on a sterile loop, placed on a glass slide and fixed by gentle heating. A Gram stain was then performed to observe the morphology.

Results: The Gram stain revealed that after day 1 (**Figure 16 A**), although organisms were spiral in shape, there were very few colonies on the plates to be utilised in experiments. At day 2 **(Figure 16 B)** however, there was sufficient growth and the organisms were still spiral. At day 3 **(Figure 16 C)** approximately 50% of organisms were coccoid and by day 5 **(Figure 16 D)** this had reached 100%. Because the colonies of *H. pylori* were becoming increasingly coccoid in form after day 2, this was selected as the optimum growth time and for all further experiments *H. pylori* were grown for 2 days on Columbia blood agar (Oxoid, UK) at 37°C under microaerobic conditions.

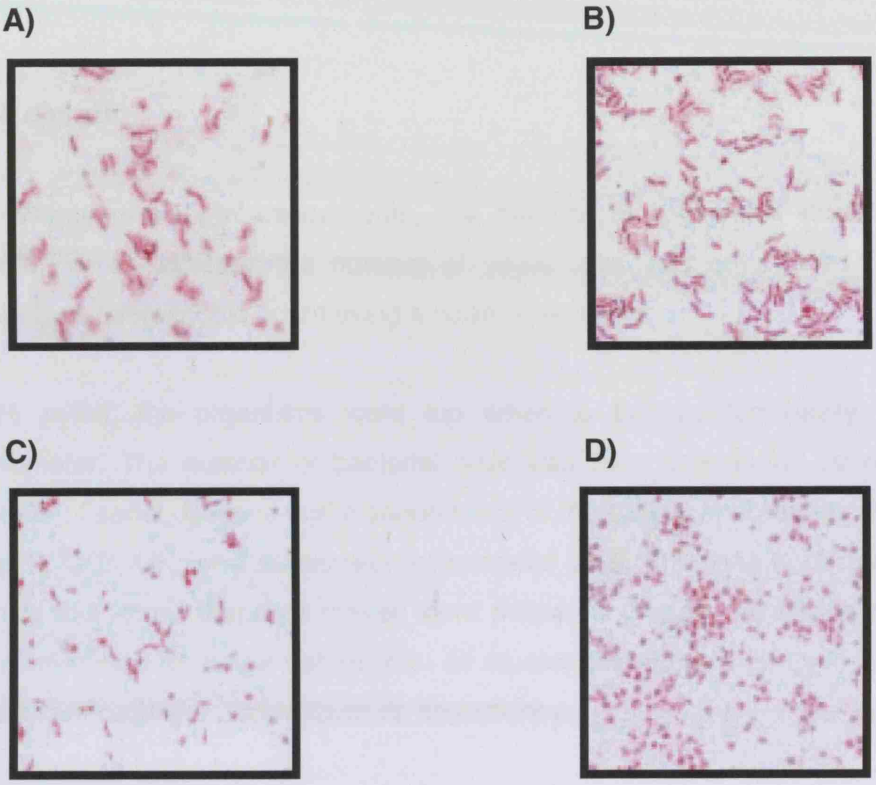

Figure 16: Gram stain showing morphology of *H. pylori* cells after different periods of growth. A) 2 days; B) 3 days; C) 4 days; D) 5 days.

2.1.1.2 *C. albicans*

C. albicans is commonly grown for 24hrs on Sabouraud-dextrose agar[307,886] or on Sabouraud-dextrose agar followed by culture in Lee's medium.[354]

Methods: *C. albicans* strain ATCC 90025 was grown on 5% horse blood agar with Columbia agar base (Oxoid, UK) and on Sabouraud-dextrose agar (Oxoid, UK) for 24hours at 37°C, to see which media was better for growth and adherence.

Results: *C. albicans* grew well on both media. However, adhesion of *C. albicans* to rat vaginal tissue sections was seen with the organisms grown on blood agar but not with those grown on the Sabouraud-dextrose agar. The optimal medium for *C.*

albicans ATCC 90025 culture was therefore chosen as blood-agar and the organism was grown on this medium for 24hrs at 37 °C for all further experiments.

2.1.2 Cell counts

For consistency of use in experiments, the number of organisms must first be determined. For *C. albicans* the number of yeast cells was determined for each experiment by a microscopic count using a haemocytometer.

For *H. pylori,* the organisms were too small to be counted easily using a haemacytometer. The number of bacterial cells was thus determined by obtaining viable counts of serial dilutions and measurement of the optical absorbance at 600nm (Ultrospec II, LKB, UK) of a suspension of bacterial cells. This was performed three times. From this, three standard curves were prepared (Figure 17) which could be used as a reference for future cell counts. All studies thereafter thus used the same concentration of organism based upon its absorbance.

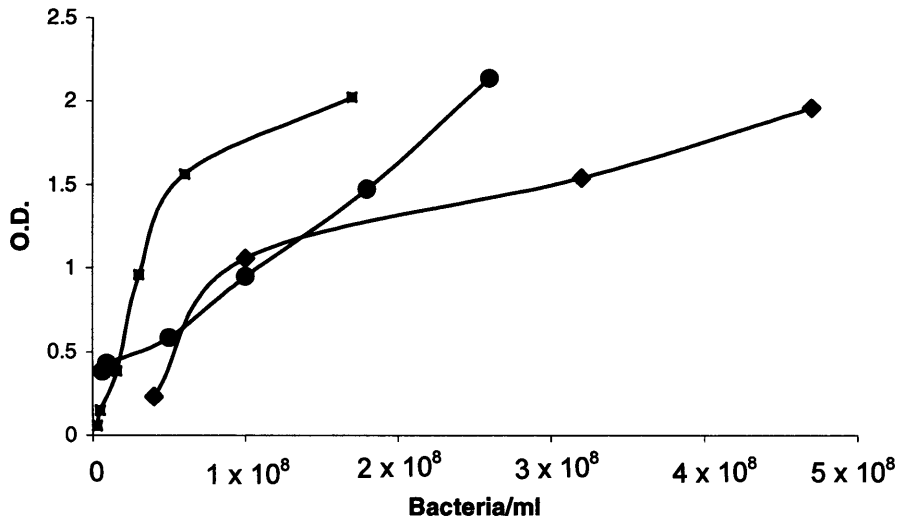

Figure 17: Standard curves showing viable counts vs optical density for *H. pylori.* Experiment was performed 3 times. O.D. = Optical density (measured in arbitrary optical density units, ODU).

2.2 Fluorescent labelling of microbes

Methods: This method was modified from Borén et al.,[15] Falk et al.[57] and Reinhard et al.[887]

To establish the optimal amount of fluorescent label required to label the organisms, suspensions of 1 x 10^9 cells/ml of each organism were made in 1ml carbonate buffer (0.15M NaCl/0.1M Na_2CO_3, pH 9.0) and different volumes of the label [10mg/ml FITC (fluorescein isothiocyanate isomer I, solution in DMSO (dimethyl sulfoxide)] were added. The suspensions were then incubated for 1hour with continuous shaking. They were then washed three times with phosphate buffered saline (PBS: NaCl 8.0g/L; K_2HPO_4 1.21g/L; KH_2PO_4 0.34g/L, pH 7.4) containing 0.05% Tween 20 (PBST) and then the pellets were resuspended in PBS. All incubation and washing steps were carried out in the dark at room temperature. The labelled microbial cells were then analysed by flow cytometry (FACSCalibur, Becton Dickinson, UK).

2.2.1 Principles of flow cytometry

(based on leaflet by BD Biosciences)[888]

Suspensions of single particles (usually fluorescently-labelled cells) are taken up into a machine (flow cytometer) and as the fluid stream passes across a beam of laser light, measurements are taken of the physical characteristics of the individual particles. The properties that are measured include relative particle size, relative granularity/internal complexity and relative fluorescent intensity. This is done by an internal optical-to-electronic coupling system, which records how the particle emits fluorescence or scatters (deflects) the incident and laser light. The extent to which light scattering occurs depends upon the physical properties of the particle (its size and density). There are two main types of light scatter that are measured by the flow cytometer: forward scatter (FSC) and side scatter (SSC). Forward-scattered light is mainly a measurement of diffracted light and is detected in the forward direction (same direction as the laser beam). It is proportional to the size or surface area of the particle or cell. Side-scattered light mostly measures refracted and reflected light (this occurs at any interface within the particle where there is a change in the refractive index). SSC is collected at approximately 90°C to the laser beam and is a measurement of the granularity and internal complexity (i.e. density) of the particle or cell.

In order to obtain measurements for a desired subset of particles, or distinguish between labelled cells and autofluorescence, an electronic threshold can be added to include only those signals that have a fluorescent intensity greater than or equal to the threshold value. A threshold can also be placed on FSC to eliminate events (particles) that are smaller than the value set as the threshold for cell size for example, debris.

A second type of definition, known as a 'gate' can be placed upon the particles in the flow cytometer, this time occurring post-acquisition of data. A gate (usally represented by 'R') is a graphical or numerical boundary that can be used to define the specific characteristics of particles which are to be included in further analysis. For example, when the sample contains a mixed population of cells, such as blood, a gate can be put on the FSC/SSC plot to allow analysis of only those cells the size of lymphocytes. A second type of gate, represented by 'M' is known as a histogram marker, and these are used to specify a range of events for a single parameter, for example, markers (M1 and M2) can be placed on the plot showing fluorescence to compare events in each marked region of the graph (marking those particles that have been labelled at a certain range of intensities), and give the percentage of cells which have labelled with fluorescence.

Results:

C. albicans

A low level of fluorescence was detected for the control (unlabelled *C. albicans*) cells (**Figure 18 A**); this represents the autofluorescence of the cells themselves. The unlabelled cells were gated (M1). When *Candida* was labelled with 10µl FITC (**Figure 18 B**) a high level of fluorescence was detected (mean Fluorescence of M2 was 968.1; arbitrary units) but two peaks were seen, indicating that not all the *Candida* cells were labelled homogenously. When 15µl FITC was used to label *C. albicans* (**Figure 18 C**), the strength of labelling was slightly stronger (mean fluorescence of M2 was 1287.7) and the second peak had slightly reduced, indicating there was slightly more homogeneity of labelling at this concentration of FITC. With 20µl FITC (**Figure 18** D) labelling was stronger (mean fluorescence of M2 was 1597.6) and labelling was much more homogeneous (only one main peak was seen). Thereafter for all experiments, 20µl of FITC was used to label *C. albicans* cells.

H. pylori

The intensity of labelling *H. pylori* was greatest when using 5µl of FITC and homogeneity remained very similar at every concentration of FITC used (**Figure 19 A-E**). Thereafter for all experiments, 5µl of FITC was used to label *H. pylori* cells.

2.3 Tissue: antigen-retrieval

H. pylori negative biopsies of human stomach (from patients with gastritis) were obtained by Professor Dino Vaira (University of Bologna, Italy) with the consent of the Ethics Committee, St Orsola Hospital, Bologna. Vaginal tissue was obtained from female Wistar rats (Charles Rivers Inc., USA). An appropriate ethical licence was obtained for the study.

In order to assess the adhesion of microbes to the epithelial surface of mucosal tissue, tissue is required in which the cell-surface molecules are exposed. When using fixed tissue, especially tissue which has been fixed in formalin, problems may occur when investigating cell surface molecules. This is because formalin or other aldehyde-based fixatives have the effect of forming intermolecular cross-links with cell surface molecules such as proteins, so that they are effectively 'masked' from detection.[889]

There are several methods which have been devised to overcome this problem, showing that the modification of protein structure by formalin is, under certain conditions, reversible. One such method is to use alternative fixatives such as Periodate-lysine-paraformaldehyde (PLP) or PLP dichromate (PLPD);[890] Zinc salts fixative (ZSF - zinc acetate and zinc chloride in a Tris-Ca acetate buffer),[891,892] which seem to result in greater preservation of antigens than aldehyde-based fixatives. Another method is to employ 'antigen-retrieval' steps before carrying out assays.

The principle of antigen-retrieval in effect is to boil the tissue sections in a particular buffer (heat induced epitope retrieval, HIER) and by doing so thermal energy is applied and the cross-links formed by the fixative are broken by hydrolysis. Alternatively, enzymes can be applied which digest the cross-links (proteolytic induced epitope retrieval, PIER). Heat or enzyme modification of the 3-D structure of the 'formalized' protein occurs, restoring it back to its original structure and the antigen is effectively 'unmasked'.[893]

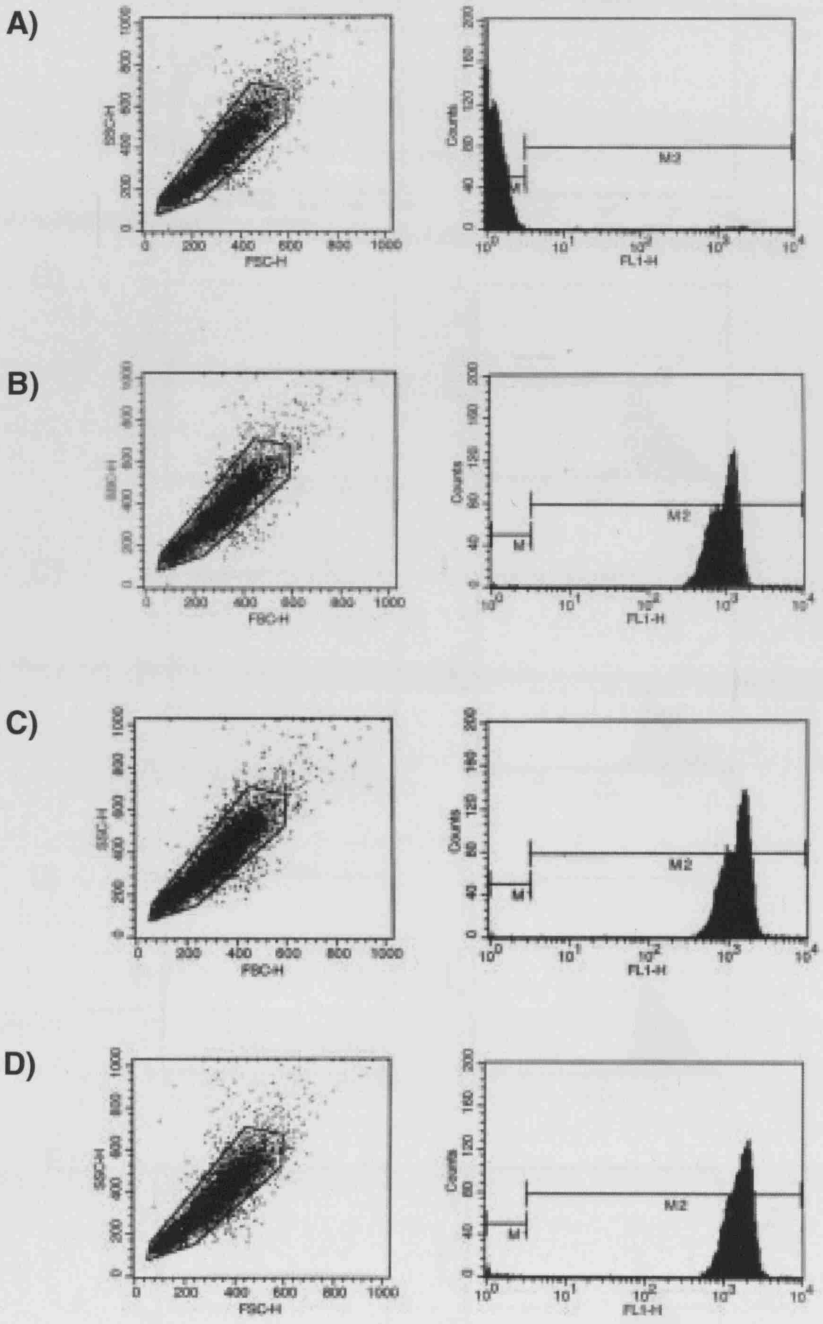

Figure 18: Labelling of *C. albicans* (1 x 10^8 cells/ml) with FITC analysed by flow cytometry. M1 = Gate on unlabelled C. albicans (autofluorescence). M2 = Gate representing any fluorescence greater than autofluorescence of the cells themselves. A) Unlabelled *C. albicans*; B) *C. albicans* + 10µl FITC (Mean fluorescence of M2 = 968.1); C) *C. albicans* + 15µl FITC (Mean fluorescence of M2 = 1287.7); D) *C. albicans* + 20µl FITC (Mean fluorescence of M2 = 1597.6).

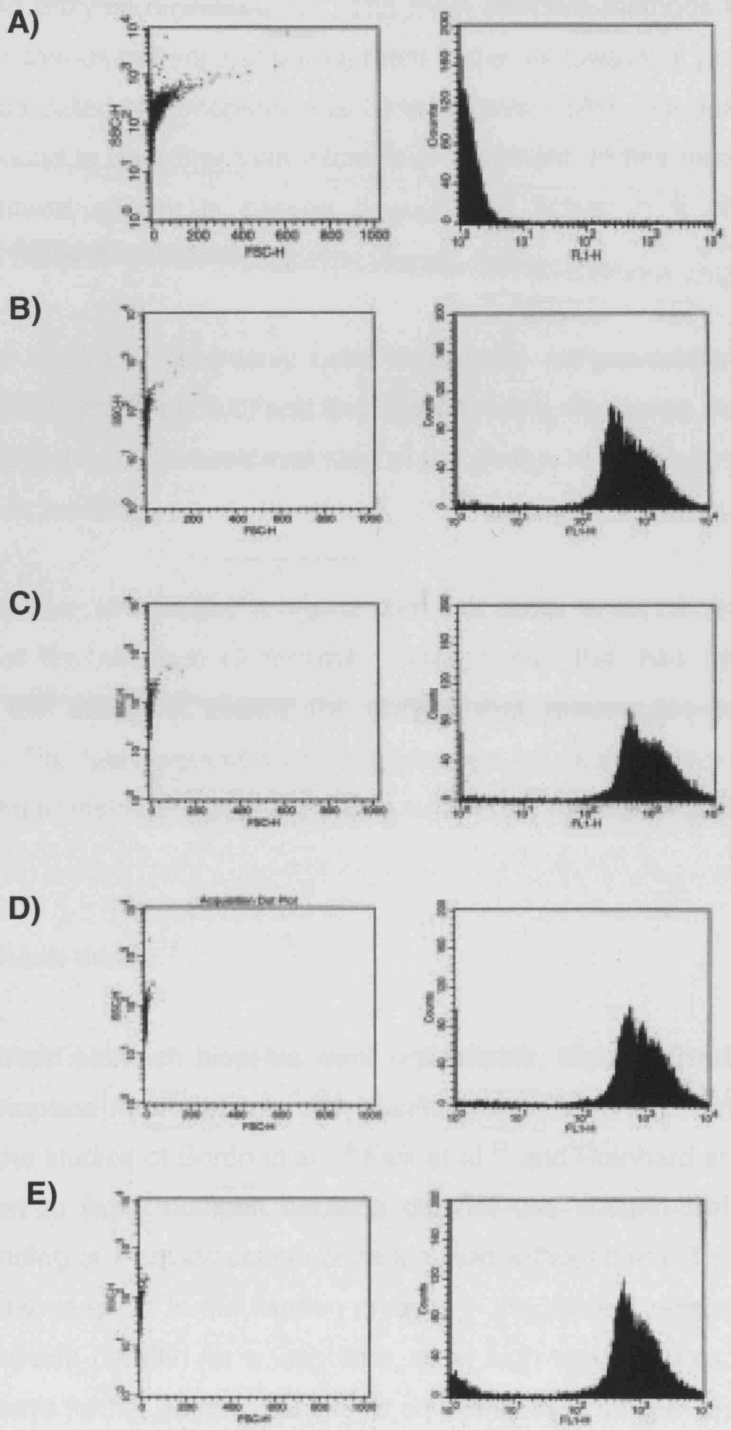

Figure 19: Labelling of *H. pylori* (1 x 10^9 cells/ml) with FITC analysed by flow cytometry. A) Unlabelled *H. pylori*; B) *H. pylori* + 1µl FITC; C) *H. pylori* + 5µl FITC; D) *H. pylori* + 10µl FITC; E) *H. pylori* + 20µl FITC.

Commonly used methods of antigen-retrieval are treatment in microwaves, pressure-cookers (to combine the action of both heat and pressure), or a combination of heat and enzyme digestion.[894,895] The most effective methods to date are placing sections in various buffers and boiling them in the microwave or pressure cooker.[896] A new method called electrochemical antigen-retrieval (EAR) has been recently devised and was found to be better than microwave treatment. In this method, an alternating electrochemical current is passed through the tissue in a chamber containing electrolyte buffer.[897]

One of the most commonly used buffers for antigen-retrieval is citrate buffer (100M sodium citrate, pH 6.0) and this was therefore chosen as the first buffer to test for optimising the antigen-retrieval step of the assay, to obtain binding of *H. pylori* to the stomach sections.

The problem of 'masked' antigens does not occur when using snap-frozen tissue because of the absence of formalin. Fresh tissue that has been snap-frozen is therefore the tissue of choice for studies that require the use of cell-surface molecules. This has been shown in many studies, for example looking at the presence of extracellular matrix antigens in healing cutaneous wounds in pigs.[898]

2.3.1 Stomach tissue

Because fresh stomach biopsies were unavailable, formalin-fixed *H. pylori*-negative stomach biopsies (from patients with gastritis) embedded in paraffin wax were used. Although the studies of Borén et al.,[15] Falk et al.[57] and Reinhard et al.[887] on adhesion of *H. pylori* to fixed stomach sections did not use antigen-retrieval steps, in this project, binding of *H. pylori* could not be attained without them. This difference can be explained by variation in the fixation process – the tissue I received may have been left to embed in paraffin for a long time, or at high temperatures; both of which are known to have further detrimental effects on cell surface antigen preservation.[891]

In order to verify whether the Lewis b or Lewis a receptors were present in the stomach biopsies, immunohistochemistry was performed on sections using anti-Lewis antibodies. The antigen-retrieval technique that gave the optimum presentation of the antigen for binding of the anti-Lewis antibodies was subsequently used for experiments optimising *H. pylori* binding to the stomach sections.

2.3.1.1 Determination of Lewis phenotype – Immunohistochemistry (IHC)

Overview of method

Tissue sections are incubated with the primary antibody, which binds to the target antigen. A secondary antibody, conjugated to an enzyme such as horseradish peroxidase (HRP) is then added and this binds to the primary antibody. The substrate used for HRP is a chromagenic substrate (diaminobenzidine, DAB) and when added to the sections, it is cleaved by the enzyme (HRP) conjugated to the secondary antibody, forming an insoluble brown-coloured precipitate at the location of the target antigen. Endogenous peroxidase is blocked at the beginning of the procedure so it does not react with the DAB causing non-specific staining.

Methods: In order to determine the Lewis phenotype of stomach tissues, it was necessary to establish the optimum antigen-retrieval method for unmasking the antigens and the amount of anti-Lewis antibody to add to the system. Experiments were performed with the aid of the histopathology department. Sections were cut (5μm) from five *H. pylori*-negative stomach biopsies (formalin-fixed and embedded in paraffin wax) and were then heat-fixed onto Vectabonded glass slides by overnight incubation at 65°C. Slides were then deparaffinised (3 mins in xylene then isopropanol), rehydrated in a graded series of alcohols, placed in racks and covered with 350mls of the antigen-retrieval solution.

The following antigen-retrieval methods were performed on each of the tissues:

1. Boiling in the microwave for 25mins in DAKO antigen-retrieval solution, pH 9.9.
2. Boiling in a pressure cooker for 25mins in DAKO antigen-retrieval solution, pH9.9.
3. Boiling in the microwave for 25mins in DAKO antigen-retrieval solution, pH 6.0.
4. Boiling in the microwave for 20mins in citrate buffer, pH 6.0.
5. Heating in 1% α-chymotrypsin/0.1% calcium chloride solution, pH 7.8 at 37°C for 10mins.

The Lewis phenotype of the tissue was then determined by standard immunohistochemical staining using the DAKO EnVision Plus HRP kit (DAKO, UK) with the primary antibodies, anti-Lewis a antigen (Le a) and anti-Lewis b antigen (Le

b), generously donated by Dr. J. Bara, Hôpital St-Antoine, Paris, France. Both antibodies were supplied at a concentration between 1-5µg/ml. For use in Lewis typing experiments, the anti-Le antibodies were diluted 1:10 and 1:50 in Tris buffered saline pH 7.4 (TBS).

Immunohistochemistry steps were as follows: all solutions were from the DAKO kit. Sections were incubated with the blocking solution for 10mins, washed for 2 mins with Tris buffered saline/0.05% Tween 20 (TBST) and incubated with 200µl of the primary antibody in a dark humid atmosphere for 1 hour. Sections were then rinsed in TBST for 2mins, incubated for 1 hour with the labelled polymer (secondary antibody-conjugated to HRP), rinsed in TBST for 2 mins and incubated with 200µl of substrate-DAB chromagen. After incubation, sections were then rinsed in TBST for 2 mins followed by warm running tapwater for a further 2 mins. Sections were then counter-stained in Harris's haematoxylin (VWR, UK) for 2 mins, rinsed in running tapwater, dipped in acid alcohol followed by another rinse in running tapwater and then dehydrated through a graded series of alcohols to xylene and mounted in Styrolite (VWR, UK).

Results: Staining for Lewis a was seen on the epithelial surface of tissue sections pre-treated in the microwave with citrate buffer, pH 6.0 and using a 1:10 dilution of the primary antibody. Therefore for all further Lewis-typing experiments, microwave boiling in citrate buffer was used to expose the Lewis a antigen. Staining for Lewis b was seen after pre-treatment in the microwave with citrate buffer pH 6.0 and also after digestion with α-chymotrypsin/0.1% calcium chloride solution, using 1:50 dilution of the primary antibody. The staining was slightly stronger after the enzyme digestion and this was used therefore to expose the Lewis b antigen for all Lewis-typing experiments in the future.

2.3.1.2 Antigen-retrieval optimisation for H. pylori adherence assay

Because boiling sections in the microwave in citrate buffer, pH 6.0 was able to expose both the Lewis a and Lewis b antigens (Lewis-typing immunohistochemistry experiments), for the adhesion/adhesion-inhibition assay, citrate buffer was used as the antigen-retrieval step of choice because the conditions would thus be kept the same when comparing adhesion to both types of tissues.

Methods: In order to establish the optimal method for exposing the cell-surface molecules required for *H. pylori* adhesion to stomach sections, 5μm sections were cut using a Leica SM2400 rocking microtome. Sections were collected on polished glass slides coated with Vectabond (Vector Laboratories, UK), deparaffinised (immersed in xylene for 3 mins followed by isopropanol, 3 mins), rehydrated by dipping through a graded series of ethanol and placed in PBS.

Sections were then placed in plastic coplin jars which held 5 slides at a time, any empty spaces were filled with clean glass slides. Each jar was filled with either 15 or 25mls of citrate buffer, pH 6.0 (100M sodium citrate tribasic dihydrate) and three jars at a time were placed evenly spaced in an 800W microwave (MW) and boiled on high power for 5 or 10 mins. After boiling, sections were cooled by placing in fresh citrate buffer for 10 mins, followed by rinsing in distilled water and placed in PBS. The control consisted of sections that were deparaffinised, rehydrated and placed in PBS without the antigen-retrieval step. The binding assay using FITC-labelled *H. pylori* was then carried out as detailed below (section 2.4). This was performed on three different Le b+ tissues using *H. pylori* that had been freshly labelled and *H. pylori* that had been frozen for three months.

Results: No binding of *H. pylori* was seen on the control sections. Sections that had been boiled for 5 mins in 15mls citrate buffer had the most adherent *H. pylori* cells **(Figure 20).** This was therefore the optimum antigen-retrieval method and was used for all experiments thereafter. *H. pylori* which had grown for more than 2 days (and was coccoid) did not bind to the stomach sections. No difference in binding was seen between the bacteria that had been freshly labelled or labelled bacteria which had been frozen for 3 months. For all further experiments bacteria were labelled and kept at –20°C until required and only bacteria that had been frozen for up to 3 months were used.

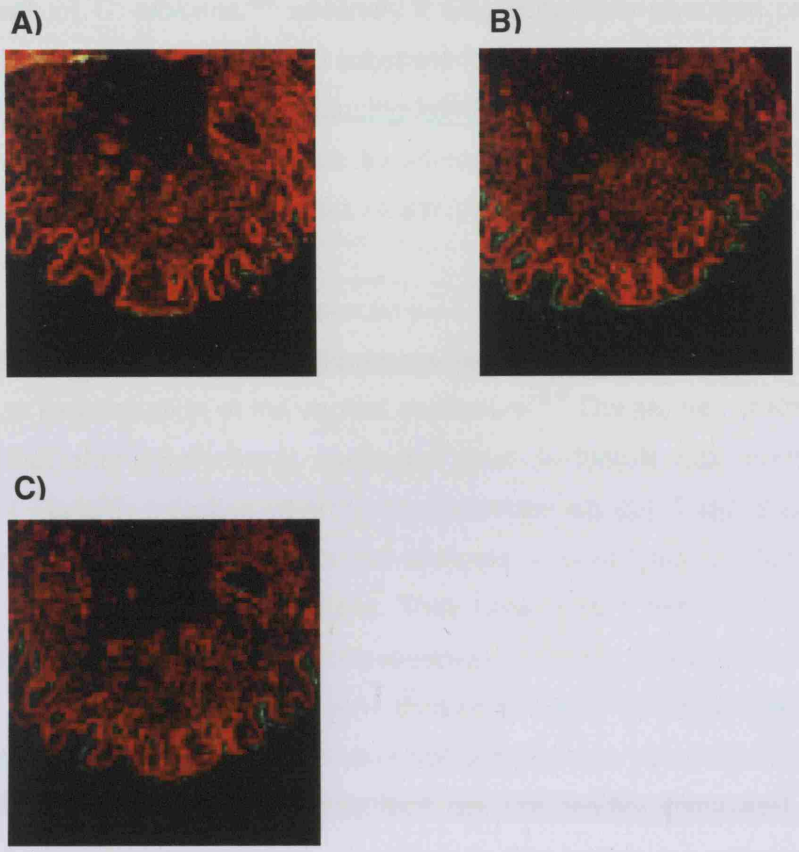

Figure 20: Effect of antigen-retrieval steps on binding of *H. pylori* to formalin-fixed stomach sections. A) Control, no antigen-retrieval step performed; B) with antigen-retrieval, 15mls Citrate buffer, 5mins MW boiling; C) with antigen-retrieval, 25mls Citrate buffer, 5mins MW boiling.

2.3.2 Rat vaginal tissue

2.3.2.1 Pseudoestrous vs non-pseudoestrous

It is known that sex hormones affect adhesion of yeasts to vaginal epithelial cells (VECs)[899] and VVC occurs most often during the luteal stage of the menstrual cycle, when levels of oestrogen and progesterone are elevated.[292] It has been shown that the elevated levels of oestrogen in particular are responsible for the binding, for example, oestrogen is required for vaginal candidiasis in mice.[295] There are three main reasons for this effect. The first is that oestrogen reduces the ability of VECs to

inhibit the growth of *C. albicans*,[292] secondly it also stimulates glycogen production which is thought to provide an attractive substrate for the yeast.[296] The third reason is that oestrogen also has the effect of inducing keratinisation of VECs[297-300] and it has been shown in oral epithelial cells that keratinised cells are more susceptible to *Candida* adhesion than those that are less keratinised or non-keratinised.[301]

As for the mouse, rats can be oophorectimised (in order to eliminate the hormonal shifts observed during oestrous-cycling) and injected with oestradiol, which has been shown to lead to keratinisation of the vaginal epithelium.[297] The studies of Kinsman et al.[900] showed that after ovariectomy, oestrogen given to female rats promoted the maintenance of *Candida* infection whereas progesterone did not. They also showed that the presence of cornified epithelium and absence of leukocytes, predisposes the rat vagina to be infected with *C. albicans*. They noted that these conditions were present at oestrous or with artificial manipulation involving ovariectomy and the administration of oestrogen (oestrogen thickened and cornified the vaginal epithelium). Infection is maintained in these conditions because yeast hyphae are able to penetrate the cornified epithelium and thus are not readily eliminated with cell turnover, and it is only with non-cornified epithelium that leukocytes can remove the yeast cells.

Epithelial cells of the superficial (cornified) layer of the mucosal epithelium may be differentiated in three main ways, depending upon what stage of the keratinisation process (i.e. cycle of cell turnover) they are in. As the epithelial cells mature, they move towards the superficial surface of the mucosa, becoming increasingly keratinised (filled with the protein keratin and aggregates called tonofilaments) as they enter the uppermost layer. Cells within this layer may be orthokeratinised (fully keratinised, dead, anucleate cells), parakeratinised (semi-keratinised, still retaining their nuclei) or non-keratinised.

The rat model of vaginal candidiasis has been well-established[243,284] and provides a useful alternative to human vaginal tissue, which is very difficult to obtain. It is for these reasons that vaginal tissue from rats has been used in this project. Because of the critical role of pseudoestrous-induced keratinisation in adhesion of *C. albicans* to the vaginal epithelium, keratinisation of the rat vagina (and hence suitability for adhesion experiments) was confirmed by two means: firstly by staining the tissue for keratin (Papanicolaou stain) and secondly by the binding of *C. albicans* to the tissue. Vaginal tissue from non-pseudoestrous rats were used for the controls alongside the pseudoestrous rats.

Methods: In order to compare adhesion of *C. albicans* to pseudoestrous and non-pseudoestrous rat vagina, vaginal tissue was obtained from pregnant female rats and from oophorectomised female Wistar rats (Charles Rivers Inc., USA) maintained in pseudo-oestrus by subcutaneous injections of 50mg oestradiol benzoate given every second day (personal communication, Professor Antonio Cassone, Istituto Superiore di Sanita, Rome, Italy). Pieces of vaginal tissue (approx. 1cm) were embedded in OCT (Tissue-Tek, UK) and 'snap-frozen' by immersing for approximately 4 mins in a beaker of hexane which had been pre-cooled to –70°C and then transferred to liquid nitogen. Sections were cut (5µm) using a cryostat (Leica CM1900, UK), an adhesion assay for *C. albicans* was performed on each of the tissues and keratinisation of the epithelial cells was confirmed by modified Papanicolaou staining.[901]

Modified Papanicolaou stain for keratin

To confirm keratinisation of the vaginal epithelium, sections were deparaffinised through two changes of xylene and rehydrated through a graded series of alcohol to distilled water, then stained in Harris's haematoxylin (VWR, UK) for 6 mins and rinsed in two changes of tap water. Sections were then dipped in acid alcohol, rinsed in tap water, dipped in ammonia water (ammonium hydroxide; Fisher Scientific, UK) and washed in running tap water for 10 mins followed by a rinse with distilled water. They were then stained in phloxine B for 5mins, rinsed in distilled water, dehydrated through a graded series of alcohol, stained in orange G6 (VWR, UK) for 5mins and rinsed in two changes of 95% alcohol. This was followed by staining in eosin-azure 50 (EA50; VWR, UK) for 4mins, two rinses in 95% alcohol and a further two rinses in absolute alcohol (dehydration). Sections were then cleared in xylene and mounted with Styrolite (VWR, UK). Control consisted of buccal epithelial cells removed by gentle scraping of the inner cheek with a swab. Cells were then placed on a clean glass slide and fixed in ethanol for 10 mins before use.

Orthokeratinised cells stain red, parakeratinised cells stain orange-red, cytoplasm of epithelial cells stains light blue-green, nuclei stain dark blue, collagen stains green-yellow and muscle stains green.

Results: *C. albicans* did not bind to the vaginal epithelium of the non-pseudoestrous rat but binding was observed on the vaginal epithelium from the pseudoestrous rat. The papanicolaou staining revealed that the vaginal tissue from the rats that were maintained in a state of pseodoestrous had a keratinised epithelium whereas in non-pseudoestrous rats, the vaginal epithelium was non-keratinised **(Figure 21).** This

explains why adhesion was observed to the vaginal epithelium of the pseudoestrous rat but not that of the non-psudoestrous rat. For all future experiments therefore, the vaginal epithelium from pseudoestrous rats was used for all *C. albicans* binding/binding-inhibition studies.

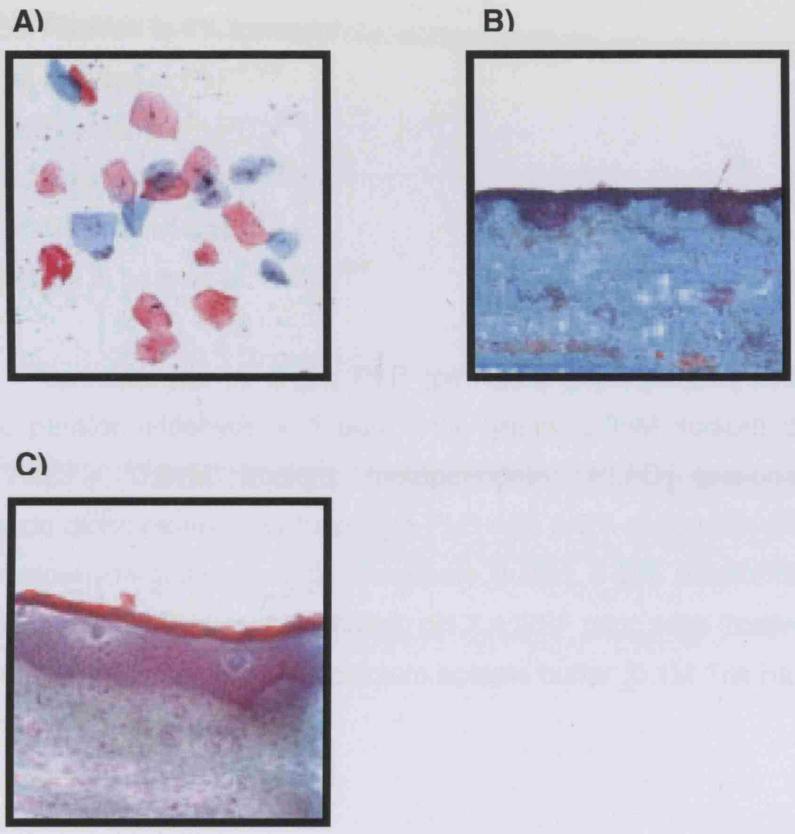

Figure 21: Papanicolaou staining showing keratinisation of epithelial cells. Red stain = keratinised cells, blue stain = non-keratinised cells. A) Control - buccal epithelial cells; B) non-pseudoestrous rat vaginal epithelium; C) pseudoestrous rat vaginal epithelium.

2.3.2.2 Processing method of vaginal tissue

Methods: Although frozen-tissue is the ideal method for tissue fixation from the viewpoint of antigen preservation, alternative fixatives to formalin have been developed which have been shown to be less detrimental to cell surface antigens. Since processing of rat vaginal tissue was carried out in-house, optimisation of tissue

preparation for adhesion of *C. albicans* was therefore able to be investigated. The vaginas from pseudoestrous rats were used for this study and processing methods compared, using a variety of different fixatives or by snap-freezing the tissue. The rat vaginas were cut into six pieces and each piece was prepared by the following methods:

1. Chemical Fixation in 4% formalin*
2. Chemical Fixation in PLP* [902]
3. Chemical Fixation in PLPD* [890]
4. Chemical Fixation in PGP* [903]
5. Chemical Fixation in ZSF* [891]
6. Snap-Frozen in hexane at –70°C [904]

*4% formalin = formaldehyde in dH_2O; PLP (periodate-lysine-paraformaldehyde) = 3parts of 16% paraformaldehyde + 1 part 0.1% lysine/ 0.05M sodium phosphate buffer, pH 7.4) + 0.01M sodium metaperiodate; PLPD (periodate-lysine-paraformaldehyde-dichromate) = Half strength PLP with 2.5% potassium dichromate; PGP (paraformaldehyde-glutaraldehyde-phosphate buffer) = 2% paraformaldehyde/ 0.05% glutaraldehyde/ 0.1M phosphate buffer, pH 7.4; ZSF (zinc salts fixative) = 0.5% zinc chloride/ 0.5% zinc acetate in Tris-calcium acetate buffer (0.1M Tris base buffer/ 0.05% calcium acetate, pH 7.4).

Tissues were fixed for six days in each fixative, processed for embedding (dehydrated through a graded series of alcohols, cleared in xylene) and then embedded in paraffin wax. Sections were cut and subsequently stained with the modified Papanicolaou stain. Adhesion of *C. albicans* (1×10^8 cells/ml) to sections from the tissues that gave optimal histological preservation was then compared.

Results: Histology was well-preserved for all the tissues, in particular with the snap-frozen, formalin and ZSF-fixed tissues. Adhesion of *C. albicans* cells to the ZSF-fixed tissue was poor whereas a lot of adherent cells were seen on the epithelial surface of the formalin-fixed and snap-frozen tissues. Because snap-frozen tissue does not involve the use of chemical fixatives, thus avoiding chemical interference with cell-surface antigens, snap-frozen tissue was chosen over formalin-fixation as the optimal method of tissue preparation. This method was therefore employed for preparing rat vaginal tissue for use in all future experiments.

Binding to the vaginal epithelium of pseudoestrous rats was only observed with *C. albicans* that had been labelled and was used on the same or next day (kept in dark at 4°C), but no binding was seen if it was used 48hrs or more after labelling. Therefore *C. albicans* was always labelled and used for adhesion experiments on the same day as labelling or the following day.

2.4 Binding, inhibition and removal assays

The binding and binding-inhibition assays were modified from that described by Borén et al.[15] Falk et al.[57] and Reinhard et al.[887]

2.4.1 Binding assay

Tissue sections were incubated for 30mins in a humid atmosphere with 200µl per slide of blocking buffer (PBST/2%BSA). Sections were then washed three times by placing in a slide rack in 350mls PBST on a rotator for 10 mins. The FITC-labelled microbes were diluted in blocking buffer to give a concentration of 1 x 10^8 cells/ml and 200µl were added to each slide and incubated in a humid atmosphere for 1hour. After the incubation, slides were washed in PBST as previously. Two hundred microlitres of propidium iodide (PI, 5µg/ml) was added and each slide incubated for 3 mins. Slides were then washed twice in PBST, air-dried and mounted with Vectashield (V-1000, Vector Laboratories, UK). All incubation and washing steps were carried out in the dark at room temperature and two tissue sections were used for each concentration of organism.

2.4.2 Binding-inhibition assay

To inhibit microbial adhesion, inhibitors were diluted in suspensions of FITC-microbe to give the required final concentrations of inhibitor and 1 x 10^8 microbes/ml. This was then incubated with continuous shaking for 2 hours at room temperature in the dark and washed with blocking buffer. The suspension was then incubated with the tissue sections as mentioned before and the subsequent steps of the adhesion assay carried out.

2.4.3 Removal assay

To determine whether the inhibitors were able to remove *H. pylori* once it had already bound to the stomach epithelium, the binding assay was carried out (as previously) but before staining the sections with PI, 200µl of inhibitor (diluted to the required concentration in PBS) were added to each section and incubated in the dark for 1 hour at room temperature. Sections were then washed three times in PBST, stained with PI, washed in PBST and mounted (as previously described).

2.5 Confocal Microscopy

Sections were observed using a Laser Scanning Confocal Microscope (Zeiss: Axiovert 100 TV) with a x10 Zeiss Plan-Neofluar objective for observing *C. albicans* on vaginal sections and a x20 Zeiss LD-ACHR objective for observing *H. pylori* on stomach sections. The excitation wavelengths were 488 (for FITC) and 568nm (for PI). 522nm and 605nm band-pass filters were used to acquire FITC emission and PI emission respectively. Digital images (512 x 512 pixels) of the sections were captured using a Biorad Lasersharp 2000 Confocal Laser Scanning System. Images were converted to .TIF files for processing. Two photographs showing adjacent areas of the tissue were taken for each tissue section.

2.6 Quantification – image analysis software

From each digital image taken, a red (PI) image showing only the tissue, a green (FITC) image showing only the microbial cells and a combined red and green image showing the tissue and microbial cells, were produced. Using these digital images, the number of adherent organisms was quantified by the optimal method devised in this thesis (ROI method with standard area method of counting) using Metamorph image analysis software (version 4.5r, Universal Imaging Corporation, USA). See Chapter 3 for details.[905]

Chapter 3

DEVELOPMENT OF QUANTIFICATION METHOD USING IMAGE ANALYSIS SOFTWARE

3.1 Introduction

A fundamental requirement in determining adhesion and inhibition of adhesion is quantification of binding. When studying binding to tissue sections, a common method of quantification that has been used is direct microscopic counts of labelled (usually fluorescent) organisms, although image analysis methods have also been applied, as direct visual counts are time consuming.

Image analysis methods have a broad application in microbiology and have been used to quantify microorganisms in geological material,[906] in food processing,[907] in the petrochemical industry,[908] for the enumeration of marine plankton[909] and biomass determination[910] as well as biomedical applications, such as analysis of bacterial motility,[911] morphology, physiological processes in bacteria following infection,[912] colony counting[913] and adhesion.[887,914]

Image analysis of adherent microorganisms is usually performed following specific stains either for DNA or some antigenic cell wall components using a fluorescently labelled antibody. Image analysis comprises three main steps: object delineation, signal to background differentiation by setting a threshold value and quantification, either by enumeration of objects or intensity of signal (usually fluorescence). Initially, a comparison of visual counts to output of the image analysis system is performed to validate the image analysis system. In dynamic systems, such a flow chamber, differentiation between static adherent organisms and free-flowing organisms have to be taken into account, usually by multiple image analysis or extended threshold values.

The image analysis system at its simplest may comprise a microscope with attached digital camera or colour-CCD device, frame grabber, computer and analysis software. The microscope may be computer controlled so that it automatically scans the required fields and in some systems focussing is also automated. Microscopes frequently used are epifluorescent or confocal laser scanning microscopes.

Various software packages have been utilised for example, NIH Image,[887] Image Pro+,[915,916] IP Lab,[917] Inducopts,[918] COMSTAT,[919] Scion,[920] Prism,[921] ICONIX[913] or written in-house.[922] When analysing adherence of irregular shaped organisms or organisms adherent to turbid solids, the accuracy of the counts can be increased by using an artificial neural network to quantify the organisms.[923]

Currently, there are a number of image analysis software packages available but no analysis of which ones are appropriate for assessing microbial adhesion to tissue sections. The aim of this study was therefore to compare current image analysis software packages in order to find the most useful one for analysing microbial adhesion to tissue sections, in particular the yeast *C. albicans* and bacterium *H. pylori*, which differ in size.

3.2 Materials and Methods

3.2.1 Tissue

H. pylori-negative biopsies of human stomach from patients with gastritis were kindly donated by Professor Dino Vaira, University of Bologna, Italy with the consent of the Ethics Committee, St Orsola Hospital, Bologna. Vaginal tissue was obtained from oophorectomised rats (Charles Rivers Inc USA) maintained in pseudo-oestrus by subcutaneous injections of 50mg oestradiol benzoate given every second day. An appropriate Ethical Licence was obtained for the study.

3.2.1.1 Stomach sections

Formalin-fixed stomach biopsies, whose epithelial cells expressed the Lewis b blood group antigen, were used. Five-micrometer sections of stomach were cut using a Leica SM2400 rocking microtome. Sections were collected on polished glass slides coated with Vectabond (Vector Laboratories, UK). After preliminary studies, the following antigen-retrieval method was used to expose the antigen. The sections were deparaffinised (3 mins in xylene then isopropanol), rehydrated in a graded series of alcohols and digested in α-chymotrypsin solution, pH 7.8 (0.1% α-chymotrypsin/0.1% calcium chloride) at 37°C for 10mins, to expose the Lewis b antigen. To expose the Lewis a antigen, after deparaffinising, the sections were boiled for 5 mins in an 800W microwave in plastic coplin jars containing 15mls of citrate buffer, pH 6.0 (100M

sodium citrate tribasic dihydrate). The Lewis phenotype of the tissue was determined by standard immunohistochemical staining using the DAKO EnVision Plus HRP kit (DAKO, UK) with anti-Lewis a (Le a) and anti-Lewis b (Le b) antibodies generously donated by Dr. J. Bara, Hôpital St-Antoine, Paris, France. Both antibodies were supplied at a concentration between 1-5µg/ml. For use in Lewis typing experiments, the anti-Le antibodies were diluted 1/10 and 1/50 for anti-Le a and anti-Le b, respectively.

3.2.1.2 Vaginal sections

Rat vaginal tissue was embedded in OCT (Tissue-Tek, UK) and 'snap-frozen'. Five micrometer sections were cut using a cryostat (Leica CM1900, UK) and keratinisation of the epithelial cells was confirmed by modified Papanicolaou staining.[901]

3.2.2 Microbial isolates

H. pylori NCTC 11637 and *C. albicans* ATCC 90025 were used in the study. *H. pylori* was grown for 2 days on Columbia blood agar at (Oxoid, UK) 37°C under microaerobic conditions.[872] *C. albicans* was grown on Columbia blood agar for 24 hours at 37°C. The number of bacterial cells was determined by measuring the optical absorbance at 600nm (Ultrospec II, LKB, UK) of a suspension of bacterial cells using the previously prepared standard curves for *H. pylori.* For *C. albicans* the number of yeast cells was determined for each experiment by a microscopic count using a haemocytometer.

3.2.2.1 Fluorescent labelling of microbes

For both organisms suspensions of 1 x 10^9 cells/ml were made in 1ml carbonate buffer (0.15M NaCl/0.1M Na_2CO_3, pH 9.0). Five microlitres of a 10mg/ml FITC (fluorescein isothiocyanate isomer I) solution in DMSO (dimethyl sulfoxide) were added to each bacterial suspension and 20µl of FITC were added to each yeast suspension. They were then incubated for 1hour with continuous shaking. The suspensions were then washed three times with phosphate buffered saline (PBS: NaCl 8.0g/L; K_2HPO_4 1.21g/L; KH_2PO_4 0.34g/L, pH 7.4) containing 0.05% Tween 20 (PBST) and then the pellets were resuspended in PBS. Labelling was confirmed by flow cytometry (FACSCalibur, Becton Dickinson, UK). All incubation and washing steps were carried out in the dark at room temperature.

3.2.3 Binding assay

Tissue sections were incubated for 30mins in a humid atmosphere with 200µl per slide of blocking buffer (PBST/2%BSA). Sections were then washed three times by placing in a slide rack in 350mls PBST on a rotator for 10 mins. The FITC-labelled microbes were decimally diluted in blocking buffer to give concentrations between $1x10^{10}$ to $1x10^{2}$ cells/ml and 200µl were added to each slide and incubated in a humid atmosphere for 1hour. After the incubation, slides were washed in PBST as previously. Two hundred microlitres of propidium iodide (PI, 5µg/ml) was added and each slide incubated for 3 mins. Slides were then washed twice in PBST, air-dried and mounted with Vectashield (V-1000, Vector Laboratories, UK). All incubation and washing steps were carried out in the dark at room temperature and two tissue sections were used for each concentration of organism.

3.2.4 Binding-inhibition assay

A binding-inhibition assay was also performed for *H. pylori* to demonstrate the application of the software to quantify inhibition of microbial adhesion.

To inhibit *H. pylori* adhesion, Lewis b-HSA (Isosep, Sweden) was diluted in suspensions of FITC-*H. pylori* to give final concentrations of 0, 10, 250, 500 and 1000µg Lewis b-HSA/ml and 1 x 10^{8} bacteria/ml. This was then incubated with continuous shaking for 2hours at room temperature in the dark and washed with blocking buffer. The suspension was then incubated with the stomach sections as mentioned before. Three tissue sections were used for each concentration. The experiment was performed twice.

3.2.5 Quantification of binding

3.2.5.1 Image capture

Sections were observed using a Laser Scanning Confocal Microscope (Zeiss: Axiovert 100 TV) with a x10 Zeiss Plan-Neofluar objective for observing *C. albicans* on vaginal sections and a x20 Zeiss LD-ACHR objective for observing *H. pylori* on stomach sections. The excitation wavelengths were 488 (for FITC) and 568nm (for PI). 522nm and 605nm band-pass filters were used to acquire FITC emission and PI

emission respectively. Digital images (512 x 512 pixels) of the sections were captured using a Biorad Lasersharp 2000 Confocal Laser Scanning System. Images were converted to .TIF files for processing. Two photographs showing adjacent areas of the tissue were taken for each tissue section.

3.2.5.2 Image analysis software

The following image analysis software packages were available for the work of this thesis and were compared:

NIH-Image (version 1.62). This is public domain software for the Macintosh and is available as a free download from the internet on the NIH's website at http://rsb.info.nih.gov/nih-image. There is also a PC version available at http://www.cs.ubc.ca/spider/ladic/executor.html

IP-Lab (version 3.6). Professional image analysis software package produced by Scanalytics Inc., USA. An evaluation version is available free on the internet on the Scanalytics website at: http://www.scanalytics.com/download/index.shtml. The full version is available for Macintosh and Windows operating systems and can be purchased from Scanalytics Inc., USA.

Image Pro+ (version 4.1). Professional image analysis software, produced by Media Cybernetics Inc., USA. This is available for Windows only.

Metamorph (version 4.5r). Professional image analysis software, produced by Universal Imaging Corporation, USA. This is available for Windows only.

3.2.5.3 Image processing and analysis

From each digital image taken, a red (PI) image showing only the tissue, a green (FITC) image showing only the microbial cells and a combined red and green image showing the tissue and microbial cells, were produced. Using these digital images four methods were devised for quantifying adherent microbial cells using each of the image analysis software packages. The steps devised for each method and software package are detailed in the Appendix.

Method 1: Counts by Merging Images

The number of microorganisms adhering to the surface of the tissue is determined by subtracting the sum of the [background (glass/non-tissue) number of microorganisms (B) + microorganisms adherent to the lamina propria (L)] from the total number of microorganisms (C). This method gives a value for only those organisms attached to the epithelial surface.

Method 2: Counts by Image Dilation

This is similar to the above method except that after carrying out the "Fill" command, the tissue image is dilated by a set number of pixels (for *C. albicans* the dilation was set to 10 pixels and for *H. pylori,* which are smaller cells, the dilation was set to 5 pixels). As before, by merging the red and green images, counts of cells in the whole image, the background and the tissue can be obtained and hence the number of adherent microbial cells calculated.

Method 3: Counts by Area

In this method, the area of at least 20 individual organisms is found using the "Wand" tool (IP Lab & NIH Image) or by using the "area" measurement (Image Pro+ and Metamorph) and the average calculated (A). Both the (red and green) and tissue (red) images are filled in with the wand or equivalent tools, so that the area with (B) and without (C) the adherent organisms is outlined. The areas of both are found. The number of organisms adhering to the epithelial surface is found by subtracting the area of the tissue image with (B) and without (C) the adherent organism, divided by the area of a single organism (A).

Method 4: Counts by Region of Interest

In this method a region of interest (ROI) is drawn by hand around the microbial cells that are directly adhering to the epithelial surface using the "wand" tool. Using the "Count" tool the number of microbial cells is provided in the highlighted region.

Metamorph has an additional "standard area" method of obtaining the counts which automatically divides clusters of microbial cells into the number of composite microbial cells by first calculating the mean size of one organism.

3.2.6 Comparison of software packages

The number of adherent microbial cells per mm of epithelial surface in the section was calculated for each of the software packages so that the results of each package could be compared. The length of the epithelial surface of each photo was calculated by outlining the tissue in the image and using the software to calculate its length (in pixels). In order to convert the units from pixels into millimeters, a digital photo of a stage micrometer on the confocal microscope was taken and using the software packages, the number of pixels represented by 1mm was obtained. All values obtained for length of epithelial surface were subsequently converted into mm.

Additionally, other data taken into consideration when comparing the software packages were the cost, availability of the package, ease of use in terms of number of manipulations, accuracy at counting the number of adherent organisms and finally which packages were able to perform the various methods listed above.

3.2.7 Operator error

In order to determine the amount of error introduced by the operator when performing quantification, epithelial length measurements and counts of adherent cells for the dynamic range for both *H. pylori* and *C. albicans* were carried out three times on three separate occasions, using the best software package and method (Metamorph with the ROI and standard area method of counting). The mean of the three counts was calculated and the standard error determined.

3.2.8 Manual method of counting

From each digital image the number of adherent cells was also counted manually using the microscope. This was performed by two independent observers and the counts obtained were averaged.

The length of the epithelial surface of each digital image was calculated manually by measuring the length of the edge of the tissue in the image (in cm). In order to convert the length obtained on the digital image into the actual length of the tissue, the image of a stage micrometer was measured manually and the number of cm represented by 1mm of the graticule was obtained. All values obtained manually for length of epithelial surface on the digital image were subsequently converted into mm.

3.3 Results

Of the four methods used to quantify adherence, only some software packages were able to perform all of them and these are outlined in **Table 6.** Of those software packages that could perform the same method of quantification, the commands were slightly different. The whole set of sequences for each method of each of the packages is given in the Appendix.

	SOFTWARE PACKAGE			
METHOD	NIH-IMAGE	IP-LAB	IMAGE PRO+	METAMORPH
Merging	–	–	*	*
Dilation	*	–	*	*
Area	*	*	*	*
ROI	*	–	*	*

Table 6: Methods of quantification that the software packages are able to perform.
- not able to be performed; * can be performed

3.3.1. Dynamic range

For both organisms the dynamic range that could be detected by the software packages was between $1x10^6$ and $1x10^9$ cells/ml **(Figures 22 and 23).** At concentrations less than $1x10^6$ cells/ml there were no adherent cells in either of the different tissue sections. Conversely, at $1x10^{10}$ cells/ml there were so many organisms on the tissue sections that it was difficult to see the epithelial surface.

3.3.2. Comparison of software packages with manual count

The accuracy of the software packages was compared to the manual method of counting used as the gold standard. The method that could be performed by all the packages (ROI method) was chosen as the comparator. Metamorph was found to

give the most accurate calculation of epithelial length, compared to the other packages and manual method (it included all the small indentations in the epithelial surface). Therefore in order to compare the ability of the packages to count the number of adherent cells, the epithelial length measurement was kept constant (i.e. the measurement obtained by metamorph was used for all methods of counting, manual and software). Counts of adherent organisms were performed three times. The results for this show that Metamorph gives the highest count of cells for both *H. pylori* and *C. albicans* over the dynamic range **(Figures 24 and 25),** with a correlation coefficient of 1.00 and 0.95 (respectively) with the manual count. The other two software packages gave correlation coefficients of 0.99 and 0.73 (NIH Image, for *H. pylori* and *C. albicans* respectively) and 0.89 (Image Pro+, for *C. albicans*).

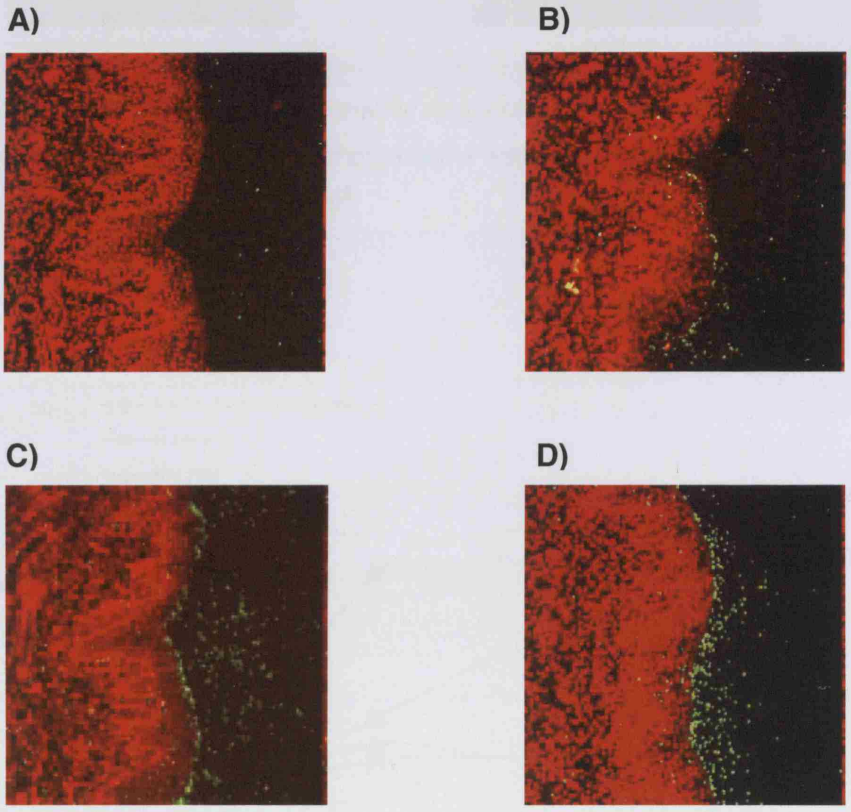

Figure 22: Confocal Images showing dynamic range of adherent *C. albicans* (cells/ml). Concentrations represent the number of organisms added to the tissue sections. A) 1×10^6 B) 1×10^7 C) 1×10^8 D) 1×10^9

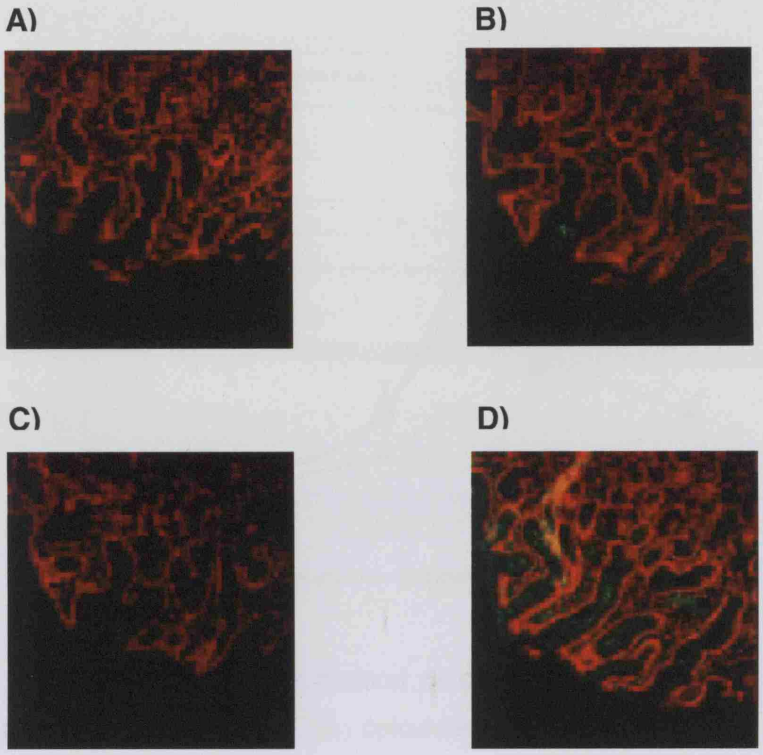

Figure 23: Confocal Images showing dynamic range of adherent *H. pylori* (cells/ml). Concentrations represent the number of organisms added to the tissue sections. A) 1 x 10^6 B) 1 x 10^7 C) 1 x 10^8 D) 1 x 10^9

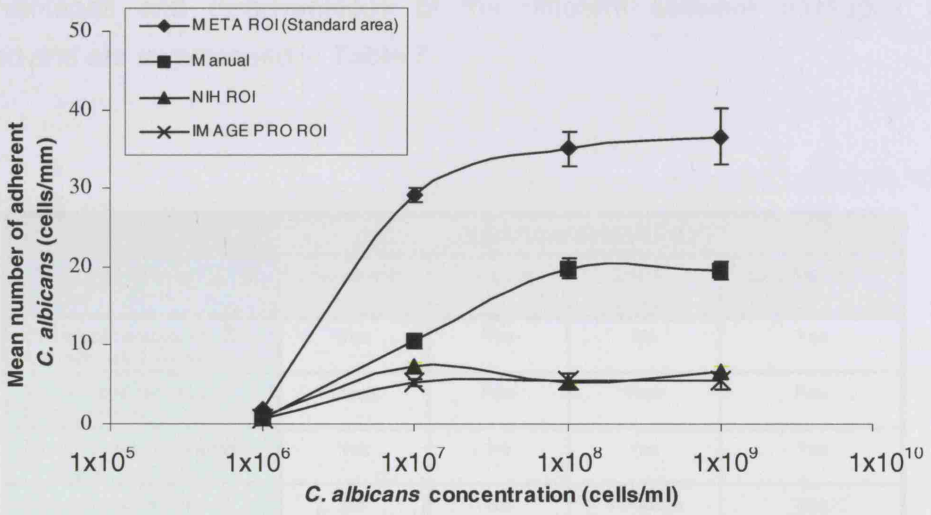

Figure 24: Comparison of manual method of counting with software packages (ROI methods), using epithelial length as calculated by Metamorph for each method. Quantification of *C. albicans* dynamic range. The mean of 3 counts is shown with error bars.

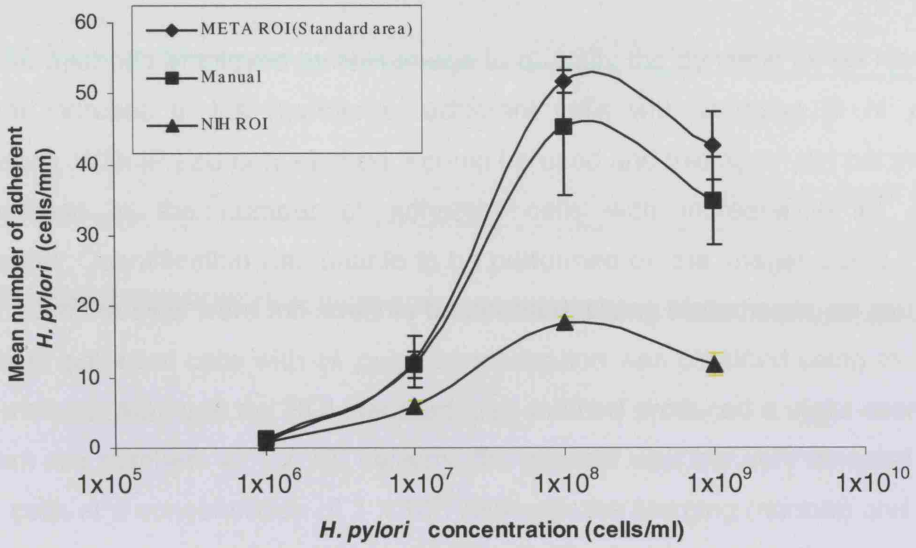

Figure 25: Comparison of manual method of counting with software packages (ROI methods), using epithelial length as calculated by Metamorph for each method. Quantification of *H. pylori* dynamic range. The mean of 3 counts is shown with error bars.

3.3.3 Comparison between the software packages

The advantages and disadvantages of the different software packages were compared and are summarised in **Table 7.**

	SOFTWARE PACKAGE			
CRITERIA	**NIH-IMAGE**	**IP-LAB**	**IMAGE PRO+**	**METAMORPH**
Can obtain counts for *C. albicans* and *H. pylori*	Yes	Yes	No	Yes
No. of steps required to obtain counts	Many	Few	Few	Few
Displays results on screen so can see if correct	Yes	No	Yes	Yes
Can separate clumps of cells	No	No	To some extent	Yes
Counts are accurate	No	No	Fairly	Very
Cost	Free	Free demo version	Expensive > £1000	Expensive > £1000

Table 7: Comparison of software packages - advantages and disadvantages.

3.3.3.1 *Helicobacter pylori* – quantification of binding

None of the methods employed by NIH-Image to quantify the dynamic range resulted in a linear increase in the number of adherent cells with increase in *H. pylori* concentration. With IP Lab only Method 3 could be used and this again did not yield a linear increase in the number of adherent cells with increase in *H. pylori* concentration. Quantification was unable to be performed on the images using Image Pro+, because the cells were too small to be counted. Using Metamorph, an increase in number of adherent cells with *H. pylori* concentration was obtained using the ROI (normal) method. Although the ROI standard area method produced a slight decrease in adherent cell numbers at 1 x 10^9 cells/ml, the number was not very different from adherent cells at a concentration of 1 x 10^8. Although the Merging (normal) and Area methods also showed an increase in adherence with concentration of bacteria, the absolute numbers of adherent cells were much lower than those obtained with the ROI method.

3.3.3.2 *Candida albicans* – quantification of binding

As for *H. pylori* none of the methods employed by NIH-Image to quantify the dynamic range showed a linear increase in the number of adherent cells with increase in concentration of *C. albicans.* IP-Lab produced the opposite result, as the concentration of *C. albicans* was increased, the number of adherent cells counted decreased. Using Image Pro+, both the dilation and ROI method resulted in an increase in number of adherent cells with increase in concentration. Metamorph, using the ROI method with standard area method of counting, was able to effectively separate clumps of cells and this was the only method that produced an increase in adherent cells with increase in *C. albicans* concentration for this software package.

3.3.4 Operator error

Only a small amount of error is introduced by the operator when quantifying the number of adherent organisms using image analysis software **(Figures 26 and 27).** The standard error ranged from 0.1 to 3.4 and all of the three counts at each concentration of organism (for both *H. pylori* and *C. albicans*) fell within two standard deviations from the mean.

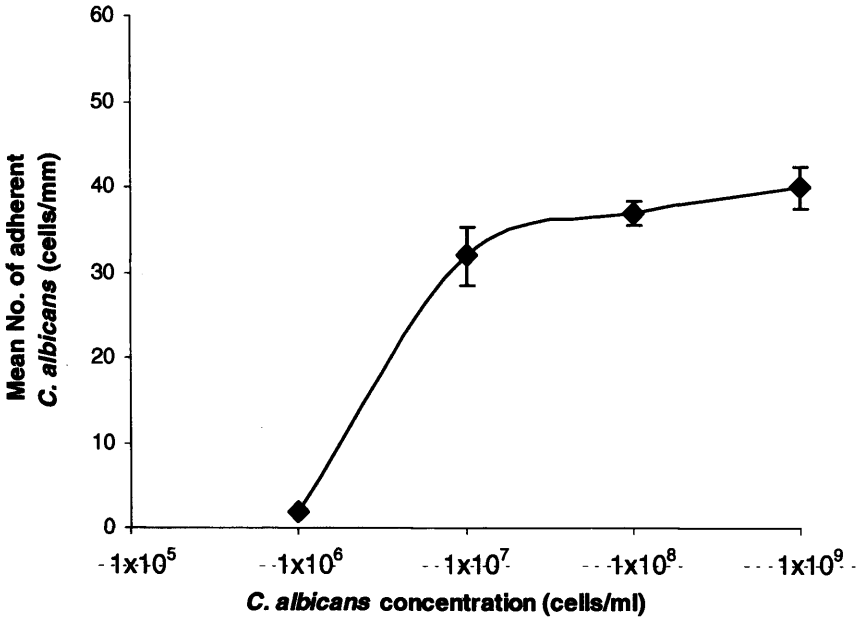

Figure 26: *C. albicans* dynamic range - operator error. Quantification was performed three times on separate occasions. The mean of the three counts is shown with error bars.

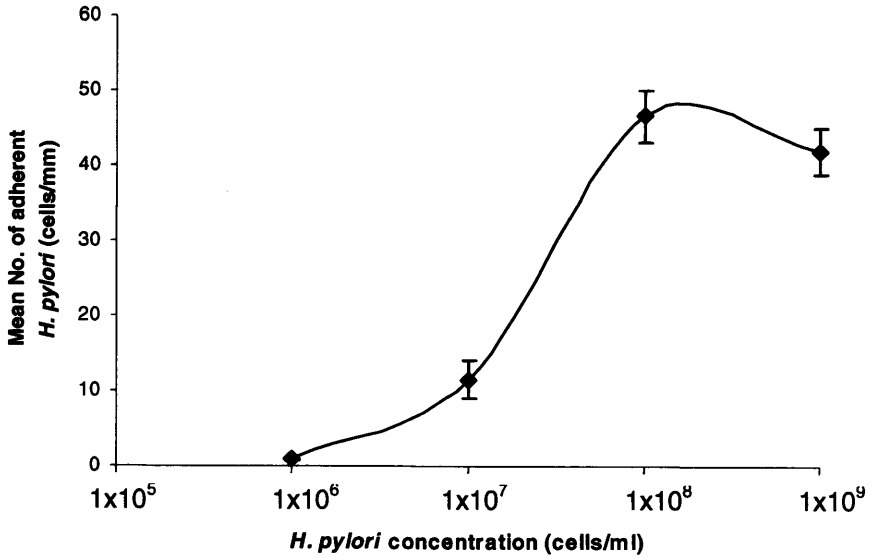

Figure 27: *H. pylori* dynamic range - operator error. Quantification was performed three times on separate occasions. The mean of the three counts is shown with error bars.

3.3.5 Inhibition of *H. pylori* adhesion by Lewis b-HSA

Using Metamorph, ROI and standard area method, the binding of *H. pylori* to Le b stomach and its inhibition with soluble Le b-HSA was investigated. The mean inhibition of binding from the two experiments was 8.3% with 10µg/ml, 48.6% with 250µg/ml, 10.6% with 500µg/ml and 19.6% with 1000µg/ml of the Le-b conjugate **(Table 8).**

% INHIBITION	Le b-HSA CONCENTRATION (µg/ml)				
	0	10	250	500	1000
Experiment 1	0	16.7	27.7	19.7	27.5
Experiment 2	0	0	69.5	1.5	11.6
Mean	**0**	**8.4**	**48.6**	**10.6**	**19.6**

Table 8: Inhibition of *H. pylori* adhesion to Le b stomach sections by Le b-HSA. The percentage inhibition of two experiments is shown.

3.4 Discussion

As a consequence of the increasing prevalence of antibiotic resistance, alternative therapeutic strategies are being sought for mucosal infections. One such strategy is the development of agents that inhibit adhesion, the primary step in the pathogenic process. Various image analysis software packages are available to quantify the adhesion of microorganisms to mucosal surfaces, but there is no published comparison of which software package is the simplest and most accurate to use. Four image analysis packages were therefore compared with a manual method of counting as a "gold-standard", using microorganisms of different sizes - a bacterium, *Helicobacter pylori* and a yeast, *Candida albicans.*

Of all the software packages tested, Metamorph (using the ROI method with standard area method of counting) was found to be the best for counting microbial adherence to epithelial surfaces of tissue sections for the two organisms employed in

this study. Image Pro+ was the second best software package. Metamorph is able to take account of clumps of adherent microbial cells into its final cell count, as is Image Pro+ (this package was used in the adhesion studies of Barthelson et al.),[915] but it does so less effectively. Moreover, Image Pro+ is unable to count *H. pylori* cells, because they are too small to be detected, which confirms Metamorph as the software package of choice. The error introduced by the investigator when performing quantification was small and thus should have very little effect on the accuracy of the resulting counts. Using Metamorph software for quantification is also better than counting manually because it is faster and more accurate at measuring both the epithelial length and the number of adherent cells, especially when they are clustered together as it is difficult to manually estimate the number of cells comprising the clusters. In none of the cases were the counts over the dynamic range linearly related to the concentration of organism added to the section. This is of course what one may expect particularly at high concentrations as receptors may be blocked and organisms may clump.

Using confocal microscopy and image analysis I have demonstrated that the binding of *H. pylori* strain NCTC 11637 to the epithelial surface of Lewis b stomach sections, can both be inhibited by the soluble glycoconjugate Lewis b-HSA and quantified by the image analysis software Metamorph. Maximum inhibition was achieved using 250µg/ml and at higher concentrations than this, inhibition was less. It may be that large amounts of glycoconjugate have an agglutinating effect on the bacteria and thus more bind to the epithelial surface and therefore inhibition is less at higher concentrations. In fact, more clumps of bacteria were seen on the epithelial surface of the tissue sections from the higher concentrations of Le b-HSA (500 and 1000µg/ml) compared to the control and lower concentrations of Le b-HSA.

The method I have devised, using Metamorph software (using the ROI method and the standard area method of counting) is a simple, quick and accurate way of quantifying adhesion and inhibition of adhesion of microbial cells to the epithelial surface of tissue sections. It is an improvement on the method developed by Reinhard et al.[887] who used NIH-Image for the same purpose. Unlike NIH-Image, Metamorph is able to separate clumps of cells into individual organisms, producing a more accurate count. I have also demonstrated that the method can be applied to organisms ranging in size from small bacteria to larger yeast cells.

SECTION III

RESULTS

Introduction

The prerequisite for most infectious diseases is adhesion of the organism to the host tissue. This essential step in the pathogenic process has therefore been subject to much investigation and consequently become the target of a new-line of antimicrobial defence: the development of anti-adhesins. Adhesion of microorganisms to host tissue is facilitated by the presence of adhesins (carbohydrates, proteins or lipids) on the surface of the organism which bind to complementary 'receptors' on the host cell surface. Anti-adhesins comprise molecules that mimic the microbial adhesin or its host cell receptor, or are antibodies that target the adhesin or receptor. These can therefore block the interaction between adhesin and receptor so that the organism is unable to bind and as a result cannot establish infection.

In the 6-20% of patients for which *H. pylori* infection results in the development of peptic ulcer disease, infection is currently treated with a combination of antibiotics and a proton-pump inhibitor (triple or quadruple therapy).[772] Treatment usually results in a high eradication rate for the organism and cure rate for the disease. However, the organism is becoming increasingly resistant to available antibiotics,[182] which poses a problem for patients with *H. pylori*-induced gastroduodenal disease, since an effective treatment would no longer exist and those with milder symptoms may be more likely to develop more severe pathology such as gastric cancer. Additionally, no vaccine or alternative treatment is currently available to prevent *H. pylori* infection.

C. albicans is also becoming resistant to current antibiotic treatment.[184] As the cause of numerous diseases in humans, this organism is an important target for which alternative therapies need to be developed. In particular, without an effective treatment for *C. albcians* infections, 75% of all women worldwide will suffer from prolonged vulvovaginal candidiasis (VVC).[204,205]

The aim of these studies has therefore been to investigate the anti-adhesive effects of several carbohydrates and domain antibodies (dAbs) against *H. pylori* infection of the stomach and *C. albicans* infection of the vagina, with the view to finding a potential new treatment for these organisms that could provide an effective alternative to failing antibiotic therapy.

Inhibitors

Domain antibodies

Domain antibodies (dAbs) are the smallest functional binding units of antibodies, corresponding to the variable regions of either the heavy (V_H) or light (V_L) chains of human antibodies.[924,925] They are less than one-tenth of the size of a full antibody (having a molecular weight of approximately 13kDa). They are designed using sequences of human germline antibodies (IgG type) and have binding sites for the superantigens Protein A or L. Protein L can naturally bind to an antibody or fragment with a V_K chain and Protein A can naturally bind to an antibody or fragment with a V_H chain. Addition of protein A or L to the dAbs can increase the binding capacity of the dAb (avidity effect) because the protein acts as a core to which the dAbs bind. As a result the dAbs are well folded and well expressed.[949]

There are several advantages of using dAbs over conventional antibodies. Because dAbs are so small, higher molar quantities per gram of product are produced, which results in a product with a significantly higher potency per dose as well as reduced manufacturing costs. The monomeric nature of dAbs means that unlike conventional IgGs which bind dimerically, they are able to bind very specifically to target receptors without causing receptor cross-linking, even at high concentrations. Their small size and compact binding sites also confers theraputic advantages such as better tissue penetration and better accessibility to less-accessibile targets such as receptor binding clefts or enzyme active sites.

Phage expression libraries allow selection and production of human single chain-variable fragments (scFv) or hypervariable single domain antibodies (dAbs) with predefined specificity, in relatively large amounts and readily standardizable.[926] Examples of therapeutic anti-*Candida* scFv applications have already been reported [927,928] but as far as is known, no human dAbs against an infectious agent have ever been generated or utilized.

Human dAbs (monomers) against the major adhesins of *H. pylori* (BabA) and *C. albicans* (Sap2, MP65 and enolase) were specifically produced for this project by Domantis Ltd., UK and tested for their ability to inhibit *H. pylori* and *C. albicans* adhesion. dAb monomers found in this study to successfully inhibit adhesion of C. *albicans* were subsequently produced by Domantis Ltd., UK as dimers (IgG or Fab-

like formats) which act as dual targeting antibodies able to bind to two adhesins simultaneously.

Production of dAbs

In order to obtain dAbs, six main steps are performed, which are summarised below.

1. Generation of recombinant C. albicans proteins.
DNA coding the sequences for the required adhesins (Sap2, MP65 and enolase) are amplified by PCR and cloned in *E. coli.*

2. Expression and purification of recombinant C. albicans proteins. Proteins from the cultured *E. coli* are purified by Nickel-chelate affinity chromatography, fractions containing the proteins are precipitated out and the proteins are refolded.

3. Generation of phage display dAb libraries. dAb phage libraries are established based on a single human antibody framework for V_H and V_L. Amino acid side-chains are diversified at positions known to make protein contacts with antigen in known molecular structures and that are naturally diversified in the mature human repertoire. These variable dAb domains are then cloned into a phage vector (pDOM4), which is the part of the phage genome containing all the necessary genes to produce infective phage particles used during the selection process.

4. dAb selection by phage display. Phages are incubated with immunotubes that have been coated with the antigens MP65, Sap2 and enolase. The tubes are washed and bound phages that remain are eluted and used to infect *E. coli.* Grown cells are recovered and used to inoculate further media for phage-growth amplification.[929] This selection process is performed two or three times and then the enriched dAb genes are recovered by digestion of purified pDOM4 phage DNA from the desired selection output with restriction enzymes. The excised dAb genes are then electrophoresed on an agarose gel and purified using the QIAgen gel purification kit. Purified digested dAb genes are then ligated with digested soluble expression vector pDOM5 using DNA ligase. Ligation product is then used to transform electrocompetent *E. coli* and the desired amount of transformed cells are plated onto agar and grown.

5. Screening by ELISA. E. coli clones are grown to express soluble dAb, centrifuged and the supernatant is analysed by ELISA – supernatant incubated with plates coated with Sap2, MP65 and enolase antigen, washed and using HRP-antibody conjugates and substrate, the bound dAbs are detected and read by a microtiter plate reader.

6. Expression and purification of dAb protein. Colonies corresponding to each soluble dAb ELISA-positive clone are grown, centrifuged and the supernatant is filtered. Supernatant of this is then mixed with Protein L-speharose (for V_L dAbs) or Protein A streamline (for V_H dAbs). The resins are recovered by centrifugation and washed in filter-plates. dAbs are then eluted with glycine and neutralised with Tris-HCl. The purity of the dAbs is then determined by SDS-PAGE, gels are stained and the dAb concentration is determined by absorbance at 280nm.

Minibodies

Minibodies are antibodies that have been genetically modified to lack the presence of the CH2 (complement-binding) region. This means that upon addition of these to an *in vivo* system they are unable to trigger the effects or functions of complement. The Fab (fragment antigen-binding) part of these antibodies is specific for BabA binding. Minibodies are therefore ideal for inhibition of adhesion experiments, because they should not lead to the killing of *H. pylori* (via the complement pathway) and so their direct interference with the adhesion of *H. pylori* to the gastric mucosa can be investigated. Also, if used *in vivo* they will not activate complement in the stomach.

Chapter 4

INHIBITORS OF *C. ALBICANS* ADHESION

4.1 Introduction

Inhibitors of *C. albicans* adhesion have been the subject of a number of investigations, especially during the past 5 years. Most of these studies have looked at oral candidiasis, attempting to inhibit *C. albicans* adhesion to buccal epithelial cells (BECs).

Inhibition of *C. albicans* adhesion to human BECs (HBECs) has been demonstrated using antifungal agents such as amphotericin B, nystatin, natamycin, miconazole nitrate, 5-fluorcytosine, rilopirox, ciclopirox amine, hexetidine (oraldene), ketoconazole, fluconazole and micafungin[930-936] anti-retroviral agents (HIV protease inhibitors) ritonavir, saquinavir, indinavir, nelfinavir and saquinavir[937,938] and the antineoplastic agents etoposide and methotrexate[934] as well as by nanoparticles, mucin, saliva and a monoclonal antibody (C7) against a *C. albicans* cell wall mannoprotein >200kDa. [408,939-941] Soluble glycopeptides and oligosaccharides from human newborn meconiums containing the H sugar sequence FUCα-1----2Galβ determinant, which is found on all blood group substances of the ABO [H] system, and the purified H (Type 2) blood group antigen, have also been shown to inhibit *C. albicans* adhesion to HBECs[373,376] as have the extracts of various plants or plant-derived materials such as propolis,[942] date[943] and the leaf of *Streblus asper* Lour (Moraceae).[944] Recently, disaccharides of the *C. albicans* fimbrial subunit [β-GalNAc(1-4)β-Gal], which mediate adherence to host cell receptors asialo-GM1 and asialo-GM2, have been shown to inhibit adhesion of *C. albicans* to HBECs.[305,375]

Of the agents mentioned above, Nystatin, Amphotericin B, Micafungin and propyl derivatives of the *C. albicans* disaccharide fimbrial subunit, have all been shown to be effective at enhancing the clearance of *C. albicans* in animals *in vivo*.[945-948] Confirmation of the effectiveness of all other agents *in vivo,* in either animals or humans, has not been reported so far.

Only three studies in the literature have investigated anti-adhesins for vaginal candidiasis, using vaginal epithelial cells (VECs). *C. albicans* adhesion to VECs has

been shown to be inhibited by N-acetyl-glucosamine, glucosamine, mannosamine and chitin.[337] However, in a competitive inhibition study, Reinhart et al.[336] showed that several of the same aminosugars (mannosamine, glucosamine and N-acetyl-glucosamine) failed to inhibit adhesion adhesion to VECs and glass beads. The authors attribute the conflicting results to differences in the study methods used. Braga et al.[931] found that sub-inhibitory concentrations of the antimycotic agents rilopirox and ciclopirox olamine significantly impaired adherence of *C. albicans* to human VECs. More recently binding of *C. albicans* to a VEC monolayer (cells isolated from human vaginal tissue biopsies) was significantly reduced (33% blocking) by prophylactic treatment of the yeast with activated lactoferrin. Activated lactoferrin was also tested therapeutically, and had a significant effect; 5mg/ml was able to detach 58% of adherent *C. albicans* cells from VECs.[403] No other *in vivo* studies using animal models (or humans) with vaginal *C. albicans* infection have tested the effects of anti-adhesive agents as a treatment for this disease.

Because VVC is such a widespread and common disease in women worldwide and the causative organism is developing resistance to current therapies, it is an important target for the development of novel treatments. This study has therefore investigated the ability of several dAbs to inhibit the adhesion of *C. albicans* to vaginal tissue sections.

4.2 Materials and Methods

4.2.1 *Candida albicans* isolates

C. albicans ATCC 90025 was used in the study and labelled with FITC. Growth conditions and labelling procedure are outlined in Chapter 2 (sections 2.1 and 2.2) and Chapter 3.

4.2.2 Vaginal sections

Sections were cut from snap-frozen pseudoestrous rat vaginal tissue. Procedures and conditions are outlined in Chapter 2 (section 2.4) and Chapter 3.

4.2.3 Inhibitors

The following inhibitors were tested for their ability to block *C. albicans* adhesion to rat vaginal sections:

4.2.3.1 Domain antibodies - monomers

Several domain antibodies (dAbs), raised against Sap2, MP65 and enolase of the type strain of *C. albicans* were used in this study. Negative controls consisted of V_H or V_K dAb dummies (ie. dAbs against irrelevant antigen) known as HEL4 or VHD. pR2 or pDOM2 refers to the expression vector routinely used by Domantis Ltd for the dAbs. For more detail, see Jespers et al.[925]

In this study, Proteins A or L were added to the dAbs because the binding ability of dAbs to their target adhesin is enhanced upon addition of these proteins, as mentioned previously.[949] This results in the formation of a polyvalent dAb, which is likely to be a more potent inhibitor of adhesion since it can target and block several adhesins at the same time. Binding capacity of the dAbs is thus enhanced.

4.2.3.2 Domain antibodies - dimers

The inhibitors that were most successful at blocking adhesion of *C. albicans* to rat vagina sections as well as clearing infection in rats *in vivo*,[950] were made into heterodimers to determine whether dimers of inhibitors would be more successful at inhibiting *C. albicans* adhesion using lower concentrations than the monomers. The dimers consisted of anti-MP65 dAb 3-6 and anti-Sap2 dAb 4A7 linked together by a 5-unit peptide linker. SM3 consisted of 3-6 linked to 4A7 and SM9 consisted of 4A7 linked to 3-6. Experiments were performed using a range of concentrations of the inhibitors and performed alongside the monomers. The controls consisted of VHD-VHD (two V_H dummies linked together). Two tissue sections were used for each concentration of inhibitor and the experiments were performed once due to the limited supply of the dimers.

The types of inhibitors used in this study and their target adhesin are summarised in **Table 9.**

INHIBITOR	TARGET ADHESIN
dAbs 4A7 and 4A14	Sap2
dAbs (x 10)	Sap2
dAbs (x 11)	Enolase
dAbs (x 7)	MP65
dAb dimers SM3 and SM9	Sap2 and MP65

Table 9: Inhibitors of *C. albicans* adhesion used in this study

4.2.4 Adhesion-inhibition assay

Details of the adhesion-inhibition assay are outlined in Chapter 2 (sections 2.4.1 and 2.4.2)

4.2.5 Quantification of binding

Sections were observed using a Laser Scanning Confocal Microscope and digital images of the sections were captured and converted to .TIF files for processing (as described in Chapter 3, section 3.2.5). Using these digital images, the number of adherent *C. albicans* were quantified using the method devised in this project (ROI method with standard area method of counting) using Metamorph image analysis software.[905] Details of the method are described in Chapter 3 and the Appendix. In order to show the percentage inhibition achieved by the specific binding region of the dAbs, all data is shown as percentage inhibition of *Candida* adhesion, with the percentage inhibition achieved by the controls (HEL4 or V_H/V_K dummies) having been subtracted.

4.3 Results

4.3.1 Monomers

4.3.1.1 Anti-Sap2 dAbs

Adhesion of *C. albicans* to rat vaginal sections was inhibited by pre-incubating *C. albicans* with the anti-Sap2 dAbs 4A7 and 4A14 **(Figure 28).** Maximum inhibition was achieved using 100µg/ml of 4A7 (61.2% inhibition) and 50µg/ml of 4A14 (58.3% inhibition). The control, HEL4/PR2 (dAb against an irrelevant antigen), at all concentrations tested, only inhibited adhesion of *C. albicans* by 5-25%. This shows the specificity of the anti-Sap2 dAbs for the Sap2 antigen and their ability to inhibit *C. albicans* adhesion to the vaginal epithelium.

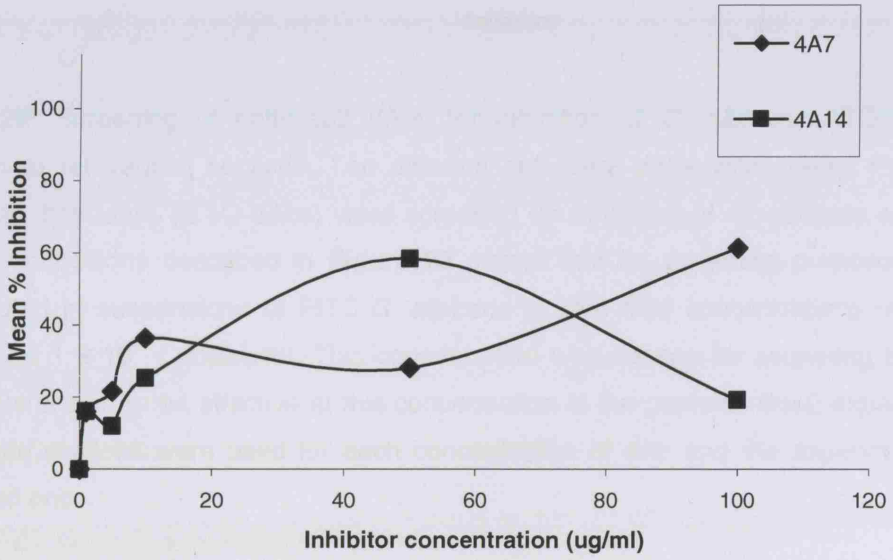

Figure 28: Inhibition of *C. albicans* ATCC 90025 adhesion to rat vaginal sections by anti-Sap2 dAbs 4A7 and 4A14. To inhibit *C. albicans* adhesion, anti-Sap2 dAbs (4A7 and 4A14) and control dAb HEL4/PR2, were diluted in suspensions of FITC-*C. albicans* (1 x 10^8 *Candida*/ml) and tested at a final concentration of 0, 1, 5, 10, 50 and 100µg dAb/ml. The mixtures were then incubated with continuous shaking for 2hours at room temperature in the dark and washed with blocking buffer, then added to the tissue sections. Three tissue sections were used for each concentration of dAb. The experiment was performed twice and the results combined. Mean inhibition is shown for each dAb.

Screening of the ten anti-Sap2 dAbs using 100µg/ml (**Figure 29**) showed that dAb 19 was the most effective giving an inhibition of 76.0%. No negative control (HEL4 / dAb dummy) was tested alongside these dAbs.

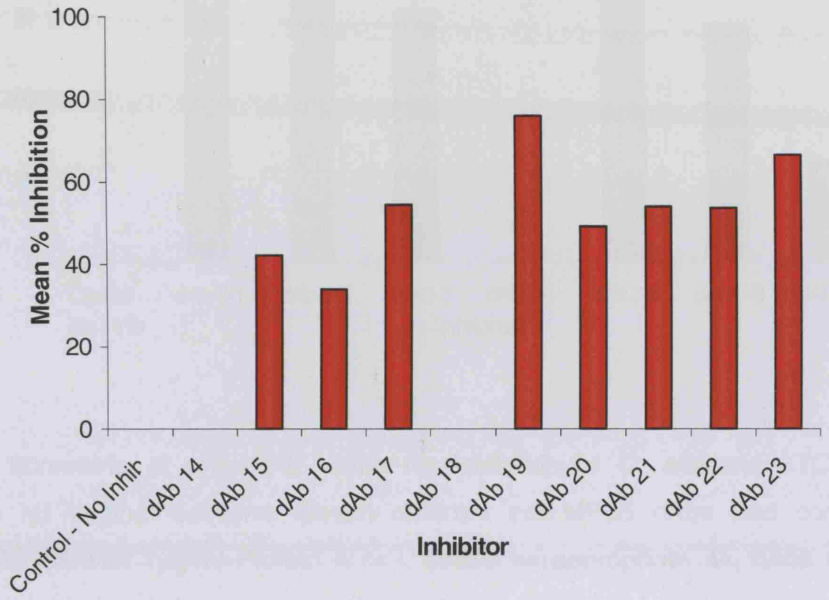

Figure 29: Screening of anti-Sap2 dAbs for inhibition of *C. albicans* ATCC 90025 adhesion to rat vaginal sections. Ten different anti-Sap2 dAbs with 1µg/ml Protein A added (as they were all V_H dAbs) were screened for inhibition of *C. albicans* adhesion using the conditions described in **Figure 28** except that for screening purposes, dAbs were diluted in suspensions of FITC-*C. albicans* to give final concentrations of 100µg dAb/ml and 1 x 10^8 *Candida*/ml. This concentration was chosen for screening because dAbs were shown to be effective at this concentration in the previous Sap2 experiments. Two tissue sections were used for each concentration of dAb and the experiment was performed once.

4.3.1.2 Anti-MP65 dAbs

Screening of the anti-MP65 dAbs (using 100µg/ml) showed that dAbs 3-1, 3-2 and 3-6 were the best at inhibiting adhesion of *C. albicans* to rat vaginal sections (Figure 30). Percentage inhibition achieved using 100µg/ml was 83.3%, 98.9% and 91.6% for dAbs 3-1, 3-2 and 3-6 respectively. The control, HEL4/PDOM2 (dAb against an irrelevant antigen did not inhibit adhesion of *C. albicans*, which confirms the specificity of the anti-MP65 dAbs for the MP65 antigen and their ability to inhibit *C. albicans* adhesion to the vaginal epithelium.

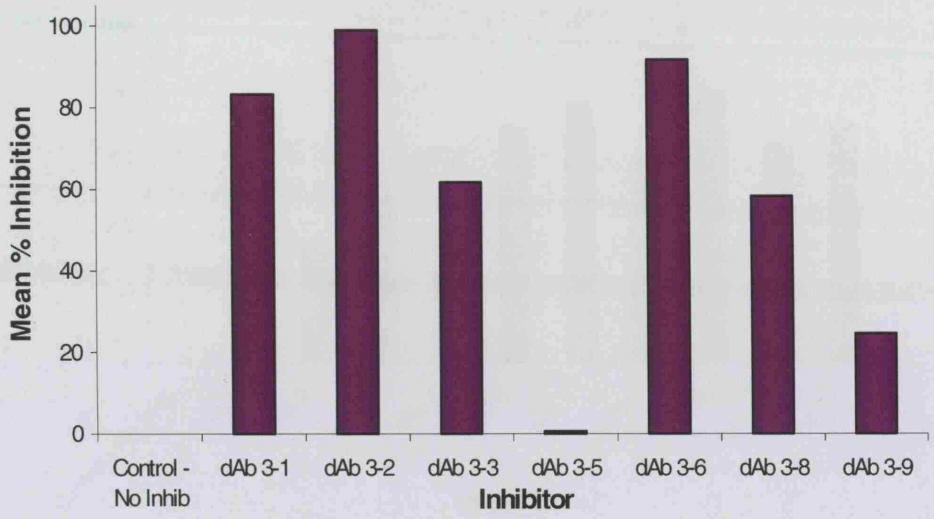

Figure 30: Screening of anti-MP65 dAbs for inhibition of *C. albicans* ATCC 90025 adhesion to rat vaginal sections. Seven different anti-MP65 dAbs and control dAb HEL4/PDOM2 [all with 1µg/ml Protein A or L added as appropriate. V_H dAbs (dAb 3-1) had Protein A added and V_K dAbs (dAbs 3-2 to 3-9) had Protein L added], were screened for inhibition of *C. albicans* adhesion using the conditions described for **Figure 28** except that for screening, dAbs were diluted in suspensions of FITC-*C. albicans* to give final concentrations of 100µg dAb/ml and 1 x 10^8 *Candida*/ml. This concentration was chosen for screening because dAbs were shown to be effective at this concentration in the previous experiments. Two tissue sections were used for each concentration of dAb and the experiment was performed once.

4.3.1.3 Anti-enolase dAbs

Screening of the anti-enolase dAbs **(Figure 31)** showed that dAbs 5, 6, 9 and 11 were the best at inhibiting *C. albicans* adhesion (% inhibition achieved was 69.7, 76.6, 77.1, 79.6 and 69.5 respectively, using 100µg/ml). The controls (V_H : dummies) did inhibit adhesion of *C. albicans,* but inhibition was much less than that of the specific dAbs themselves (21.4% mean inhibition). This shows that the dAbs were specific for the enolase antigen and that only a minor part of their ability to inhibit *C. albicans* adhesion to the vaginal epithelium is due to non-specific regions of the V_H or V_K domains.

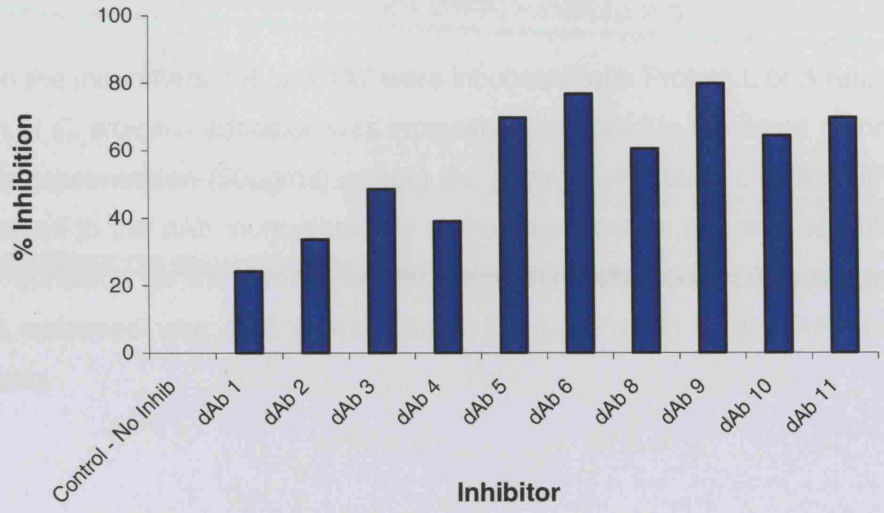

Figure 31: Screening of anti-enolase dAbs for inhibition of *C. albicans* ATCC 90025 adhesion to rat vaginal sections. Eleven different anti-enolase dAbs and control V_H dummy (all dAbs were V_H dAbs and had Protein A added) were diluted in suspensions of FITC-*C. albicans* to give final concentrations of 100µg dAb/ml and 1 x 10^8 *Candida*/ml and screened for inhibition of *C. albicans* adhesion using the conditions described in **Figure 28**. This concentration was chosen for screening because dAbs were shown to be effective at this concentration in the initial anti-Sap2 dAbs experiments and100µg/ml was the highest concentration able to be obtained from the sample of dAbs available. Two tissue sections were used for each concentration of dAb and the experiment was performed once.

4.3.2 Dimers

Adhesion of *C. albicans* to rat vaginal sections was inhibited by pre-incubating *C. albicans* with the anti-*C. albicans* dimer dAbs SM3 and SM9 **(Figure 32).** Maximum inhibition was achieved using 10µg/ml of SM3 (74.7% inhibition) and 50µg/ml of SM9 (75.0% inhibition). Inhibition of adhesion using the monomers (without Proteins A or L) was lower than that of the dimers at all concentrations tested; maximum inhibition was 39.4 and 45.4 for dAbs 3-6 and 4A7 respectively using 50µg/ml). This is likely to be because there was no addition of Proteins A or L in the earlier studies. The controls HEL4 and VHD-VHD also inhibited adhesion of *C. albicans* at all concentrations tested but inhibition was nearly always lower than the dimers and this value was

subtracted from the percentage inhibition of the inhibitor dAbs to give the overall percentage inhibition of the specific binding regions of the inhibitor dAbs themselves.

When the monomers 3-6 and 4A7 were incubated with Protein L or A respectively, inhibition of *C. albicans* adhesion was increased compared to the same monomers at the same concentration (50µg/ml) without the addition of Protein L or A. With protein A or L added to the dAb monomers, the monomers were as effective at inhibiting *C. albicans* adhesion as the dimers (at the same concentrations, 50µg/ml); maximum inhibition achieved was 53.5% and 73.6% for 3-6+Protein L and 4A7+Protein A respectively.

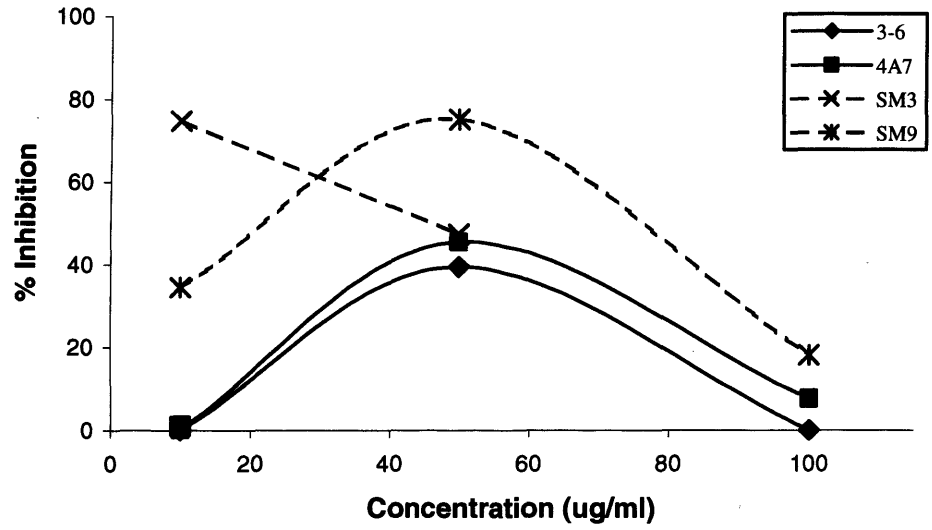

Figure 32: Screening of anti-*C. albicans* dimer dAbs for inhibition of *C. albicans* ATCC 90025 adhesion to rat vaginal sections. Two different dimer dAbs (SM3 and SM9) were tested alongside the monomer dAbs (3-6 and 4-6). Controls consisted of HEL4 for monomers and VHD-VHD (V_H dummy linked to V_H dummy) for dimers. All dAbs and controls were diluted in suspensions of FITC-*C. albicans* to give final concentrations of 0, 10, 50 and 100µg dAb/ml and 1 x 10^8 *Candida*/ml and screened for inhibition of *C. albicans* adhesion using the conditions described in **Figure 28**. Two tissue sections were used for each concentration of dAb and the experiment was performed once. For each dAb the percentage inhibition is shown from which the percentage inhibition achieved by their relevant controls has been subtracted.

The results of all the *C. albicans* inhibition studies are summarised in **Table 10.**

INHIBITOR (dAb)	TARGET ADHESIN	MAX. % INHIBITION	CONCENTRATION (µg/ml)
MONOMER EXPERIMENTS			
4A7*	Sap2	61.2*	100
4A14*	Sap2	58.3*	50
4-19**	Sap2	76.0**	100
3-1	MP65	83.3	100
3-2	MP65	98.9	100
3-6	MP65	91.6	100
5	Enolase	69.7	100
6	Enolase	76.6	100
9	Enolase	79.6	100
11	Enolase	69.5	100
DIMER EXPERIMENTS			
SM3	MP65-Sap2	74.7	10
SM9	Sap2-MP65	75.0	50
3-6	MP65	39.4	50
4A7	Sap2	45.4	50

Table 10: Summary of results of *C. albicans* inhibition experiments, showing the best inhibitors tested in each group. Unless indicated by * the data represents % inhibition achieved by dAbs to which the relevant proteins A or L were added. ** Indicates no control was tested or deducted from the data.

4.4 Discussion

at least once per lifetime
C. albicans is the cause of VVC in 75% of all women worldwide, which can be a distressing and recurrent disease. VVC is currently treated by using antibiotics taken either orally or applied topically,[951] however, the yeast is becoming resistant to the antimycotics available. By using dAbs as anti-adhesins, (which block adhesion of the yeast to the vaginal epithelium), it may be possible to develop an effective and alternative treatment for this disease and offer a solution which overcomes the problem of the development of resistant organisms.

Testing of the anti-*Candida* dAbs showed that anti-Sap2 dAbs 4A7, 4A14 as well as anti-MP65 dAbs 3-1, 3-2 and 3-6 and anti-enolase dAbs 5, 6, 9 and 11 are effective inhibitors of *C. albicans* adhesion to the rat vagina *in vitro.* These dAbs are therefore probably the most specific of the selection for the binding region of the adhesins they were raised against. Although anti-Sap2 dAb19 also showed a high percentage inhibition of *C. albicans* adhesion, because no negative control dAb was tested alongside it, the exact effectiveness of this dAb at inhibiting *C. albicans* adhesion is not certain.

In the present study, two of the dAbs that were most successful at inhibiting adhesion of *C. albicans* to rat vaginal sections (anti-MP65 dAb 3-6 and anti-Sap2 dAb 4A7) were made into heterodimers, consisting of both dAbs joined by a 5-unit peptide linker. These were tested alongside the monomers in an inhibition experiment (all without protein A or L added). At nearly all the concentrations tested, the maximum inhibition of *C. albicans* adhesion by the dimers was found to be higher than the maximum inhibition of the monomers and the dimer controls (VHD-VHD), showing that the dimers were more effective than the monomers at inhibiting *C. albicans* adhesion to the vaginal tissue sections, probably because they target two *C. albicans* adhesins simultaneously. However, the controls HEL4 and VHD-VHD (dAbs against an irrelevant antigen) also inhibited adhesion of *C. albicans* at all concentrations tested, indicating that the inhibiting ability of the monomers and dimers was probably caused in part by non-specific binding of the dAbs to the target adhesins of *C. albicans.* It may also be that they reduce *C. albicans* adhesion by interfering with electrostatic interactions, as shown by their ability to inhibit adhesion to plastic.[950]

The percentage inhibition achieved by the 4A7 and 3-6 monomers was higher when they were tested in the earlier monomer studies than when they were tested alongside the dimers. In the case of dAb 3-6 this was probably because in the first

experiment Protein L was added; it is known that addition of the protein enhances the binding ability of the dAbs because the protein is able to bind to the dAbs thus forming a core around which the dAbs bind. This results in the formation of a polyvalent inhibitor which is likely to be a more potent inhibitor of adhesion since it can target and block several adhesins at the same time. The difference seen between the experiments involving dAb 4A7 may simply be due to variation in receptor expression on the tissue section and fall within the error margin of the experiments.

The results from studies involving the same dAbs in an *in vivo* study of rat candidiasis,[950] correlate closely with those found in the present *in vitro* study. Clearance of the organism from the vagina was observed in animals treated intravaginally (both prophylactically and therapeutically) with the same dAbs shown to be effective in the *in vitro* study (anti-Sap2 4A7 and 4A14 as well as anti-MP65 3-1, 3-2 and 3-6). Some confidence can therefore be placed in the ability of the *in vitro* model to predict and select inhibitors of adhesion that will also be effective *in vivo*. The *in vitro* results also strongly suggest that the clearance of the organism from the vagina of the rats treated with the dAbs in both the preventative and the therapeutic mode is due to the specific anti-adhesive properties of the dAbs. This encourages the idea that *in vitro* studies with human vaginal tissue will help in the screening and selection of potential inhibitors of human *Candida* infection.

DeBernardis et al.[950] have also shown that some of these dAbs (anti-MP65 dAbs 3-1, 3-2, 3-6) are able to inhibit adhesion of *C. albicans* to plastic (polystyrene) and epithelial cells (human umbilical vein epithelial cells, HUVEC). This, combined with their ability to inhibit adhesion to the rat vaginal epithelium, suggests that apart from specific inhibition of adhesin-receptor interactions, the dAbs seem to have a disruptive effect on the surface of the *Candida* cells, interfering with the hydrostatic or electrostatic forces that are known to be involved in the adhesion of *Candida* to plastic materials.[952] Adhesion of *C. albicans* to plastic has been shown to be mediated by cell wall mannoproteins[41] and monoclonal antibodies against *C. albicans* cell surface mannoproteins have been found in past studies, to inhibit adhesion of the organism to plastic.[953,954]

This study is of importance because although inhibitors of *C. albicans* adhesion to the vaginal epithelium have previously been shown,[403,931] the use of dAbs is a novel treatment. Previous studies also involved the use of VECs rather than tissue sections (or animal models) which were used in this study and therefore the results of this study provide a much more accurate representation of the human *in vivo* situation.

Although the anti-adhesive effects of the anti-*Candida* dAbs have been shown *in vitro* and *in vivo* in rats,[950] further studies have to be carried out investigating their effects in humans *in vivo.* The fact that the dAbs have been shown to clear *Candida* infection in rats *in vivo* shows that they are able to survive the digestive process and that they have the potential to be an effective therapeutic treatment in humans against vaginal candidiasis. Additionally, since the dAbs are able to inhibit adhesion to epithelial cells and plastic, they may also prove to form the basis of an effective preventative measure against *Candida* biofilm formation on plastic devices in nosocomial *Candida* infections.

The results of the present study suggest that dimers of dAbs are more effective than the monomers at inhibiting *C. albicans* adhesion, but further experiments are required to confirm this. It would be interesting in future experiments to investigate the effectiveness of combinations of the dAbs and to further elucidate the effectiveness of dimers or even trimers of the dAbs.

In conclusion, this study has shown the effectiveness of several dAbs against *C. albicans* adhesion to the vaginal epithelium both *in vitro* as well as *in vivo* and suggests that using dAbs is very likely to be an effective treatment for VVC in humans.

Chapter 5

INHIBITORS OF *H. PYLORI* ADHESION

5.1. Introduction

A number of studies in the literature have attempted to inhibit the adhesion of *H. pylori* to the gastric mucosa using a variety of different agents (details of the studies, inhibitors and target adhesins/receptors, are summarised in **Table 11**). The majority of investigations have targeted the BabA and NLBH (HpaA) adhesins of *H. pylori*, probably because the adhesin-receptor interactions of these adhesins have been fairly well characterised. Most of the investigations were performed *in vitro,* using gastric cell lines (monolayers) or immobilised receptors as their model of the mucosa The problem with using cell lines and monolayers is that they are not a very good representation of the *in vivo* environment, since they comprise of only one homogeneous cell type, although this does allow for reproducible results. Additionally, since most of the cell lines used in these studies were taken from gastric tumours, the pattern of cell surface receptors is likely to be different to the normal gastric mucosa, since cancer cells express their own set of receptors,[955] again posing a limitation on the accuracy of studying host-microbe adhesin-receptor interactions in these models.

Instead therefore, several investigators have used tissue sections from the gastric mucosa of human stomachs.[15,57,621,887,905] This is a better model for studying adhesin-receptor interactions because the gastric epithelial cells are *in situ* with all the other mucosal cell types and the true pattern and types of receptors expressed in the clinical situation are present. Although the best way of studying the effects of microbial inhibitors of adhesion is to use *in vivo* models (animals or preferably humans in clinical trials), which several studies have done,[956-961] only a handful of studies which found successful inhibitors *in vitro* have gone on to test these agents *in vivo.*

Unfortunately most of the inhibitors that showed promise in *in vitro* experiments and were also tested *in vivo,* were found to have a low success rate in humans and failed to cure infection. The limitations of the studies were the need to test different doses of inhibitors and use longer regimes of treatment. All of the studies require larger cohorts of patients to be able to accurately determine the efficacy in humans, which is expensive and requires long-term investigation, although this is a

requirement if potential inhibitors are ever going to be used as therapeutic agents in humans. Additionally, agents that were successful in *in vitro* studies but not in clinical trials were probably because the *in vitro* studies used non-ideal models, such as cell lines, for testing. Interestingly, successful inhibitors found by testing on tissue sections were also shown to have some success (and inhibit adhesion) when tested *in vivo* (for example Le b, sLe x and immunoglobulins from colostrum; see **Table 11**). Although many studies have investigated inhibitors of *H. pylori* adhesion, because most of these were unsuccessful or had limited success *in vivo*, further inhibitors still need to be found which will be successful *in vivo*.

It is with this in mind that the present study has used *in vitro* models of adhesion, using human gastric tissue sections so that a more accurate indication of potential inhibitors of adhesin-receptor interactions can be gained, which can also be achieved in a relatively short period of time. Additionally, due to problems with the quantification of adherence, image analysis software (previously tested, see chapter 3) was employed to increase the accuracy of analysing the effects of the inhibitors. As the literature has shown, because *H. pylori* adhesion to the gastric mucosa involves multiple adhesins,[609] which most likely operate simultaneously, by using agents that target only single adhesin-receptor interactions at a time, complete inhibition of adhesion is rarely achieved. It is for this reason that the most successful inhibitors found in this study were also tested in combination in an attempt to produce an increase in efficacy, by targeting more than one adhesin at a time.

Moreover, even fewer studies in the literature have investigated the ability of these compounds to detach *H. pylori* once it has already bound to the gastric mucosa, which would be more useful as an indicator of its efficacy as a possible treatment for *H. pylori*–infected patients. Simon et al.[665] found that 3'SL was able to detach *H. pylori* cells from the stomach of rhesus monkeys and Hata et al.[964] showed that the ganglioside GD_3 and glycosphingolipid sulfatide, both removed bound *H. pylori* from gastric adenocarcinoma cell monolayers. The present study has therefore investigated the ability of inhibitors (dAbs, minibodies and carbohydrates) to inhibit the adhesion of *H. pylori* to stomach sections and more importantly their ability to remove the organism once it has already bound to the mucosa. A few studies have investigated the effects of antibodies as anti-*H. pylori* adhesins, however, the use of dAbs and minibodies are novel treatments.

Table 11: Studies in the literature showing successful inhibitors of *H. pylori* adhesion.

Inhibitor	Target adhesin /receptor	Study Type	Gastric Tissue model	Study Method	*H. pylori* strain	Effective concentration	Result & epitope responsible for inhibition	Reference
Target: BabA adhesin								
scIgA from human colostrum; **Le b-HSA, Le y** and **H1** oligosaccharides	BabA adhesin	*In vitro*	Tissue sections (normal human stomach)	Inhibition-adhesion assay to tissue sections; analysed by microscopy	2 clinical isolates (GU and acute gastritis respectively)	20µg/ml of Le b-HSA, Le b or H1 antigen; 10µg/ml of Le b+ human colostrum	93%; 78%; 100%, 100% and 48% for Le b-HSA, scIgA,Leb+ colostrum. Le b and H1 antigen respectively.	15
Milk (fat removed) from Le b-producing goats	BabA adhesin	*In vitro*	Le b antigen	Inhibition of adhesion to Le b antigen	NCTC 17875	25-fold diluted milk	83% inhibition	549
Le b-HSA	BabA adhesin	*In vitro*	Le b+ Tissue sections (human stomach with gastritis)	Inhibition-adhesion assay to tissue sections; analysed by microscopy and quantified by image analysis software	NCTC 11637	250µg/ml with 1 x 10^8 Hp/ml	48.8% inhibition of adhesion	905
Bovine submaxillary mucin, fetuin, asialofetuin, bovine and **human sialyllactose, human serum IgA** and **human secretory IgA**	BabA adhesin??	*In vitro*	Tissue sections (normal human stomach)	Inhibition-adhesion assay to tissue sections; analysed by microscopy	2 clinical isolates (GU and acute gastritis respectively)	Bovine submaxillary mucin (500µg/ml); human secretory IgA (15µg/ml)	No Inhibition of adhesion with calf serum fetuin, asialofeutin, human serum IgA, human and bovine sialyllactose. 100% inhibition with bovine mucin, human colostrum scIgA. **Fucosylated** not **sialylated glycoprotein responsible**	57
Le b, Le a, sIgA and **scIgA** (from colostrum of Le a or Le b humans)	BabA and other ?? adhesins	*In vitro*	Le b+ Tissue sections (human stomach)	Inhibition-adhesion assay to tissue sections; analysed by microscopy and quantified by image analysis software	NCTC 11637	10 and 50µg/ml Hp unknown conc	Inhibition to Le b stomach using 50u/ml, was 96% (Le b), 65% (Le a), 49% (scIgA-Le a human), 68% (sIgA) and 79% (20µg/ml scIgA-Le b human). No inhibition to Le a stomach	887
Target: SabA adhesin								
Porcine milk from sLe x and Le b expressing or non-expressing pigs	BabA and SabA adhesins	*In vitro* and *in vivo*	*In vitro*:J99, CCUG 17875 (sLe x) and 17875 (Le b). *In vivo:* counted	J99*In vitro:* inhibition of adhesion to Le b and sLe x neoglycoconjugates. *In vivo:* Oral administration to infected mice; colonies in stomach grown and	4mls milk daily for 9 days	Transgenic FVB/N mice expressing human fucoslyated blood group antigens	*In vitro:* Inhibition of adhesion 50-90% for all strains to all neoglycoconjugates. *In vivo:* both types ofmilk reduced colonisation and was significant reduction using sLe x and Le b+ milk. **Epitopes in milk= sLe x and Le b**	962

Table 11 continued

Inhibitor	Target adhesin /receptor	Study Type	Gastric Tissue model	Study Method	*H. pylori* strain	Effective concentration	Result & epitope responsible for inhibition	Reference
Target: SabA adhesin								
sLe x and **sLe a**	SabA adhesin	*In vitro*	Hp+ inflamed human gastric biosies	Inhibition-adhesion assay to tissue sections	BabA mutant of NCTC 17875	Unknown	Pretreatment of Hp with sLe x inhibited adhesion by approx. 90%. Pretreatment of tissue sections with anti-sLe x mAb inhibited adhesion by approx. 72%.	621
Target: HpaA (NLBH) adhesin								
NLBH-derived peptide, **anti-peptide Ab, anti-adhesin anti-serum** and **fetuin**	HpaA (NLBH) adhesin and NeuAcα-2,3Galβ1,4Gluc	*In vitro*	Hep2 cells ,ATCC CCL-23 (Laryngeal carcinoma)	Inhibition-adhesion assay to cell monolayers in 12-well plates; assessed by microscopy	ATCC 43526 and 2 clinical isolates	Ppeptide = 0-1600ng; anti-peptide Ab = 0-120ng; anti-adhesin serum = 0-180ng; fetuin = 0-80ng	All inhibitors significantly reduced adhesion	659
BIC (bovine immunoglobulin colostrum preparation), **3'SL** (3'Sialyllactose) and **rLF** (recombinant LF) from milk of Hp-immunised cows	HpaA for 3'SL others unknown	Clinical study	Hp+ adults	Oral administration, 3days.	n/a	BIC = 7 x 7.6 or 7.8g/ml doses for 2days; 3'SL = 5 x 2g for 1 day; rLF = 5 x 250mg or 1g for 1 day	None cleared infection	956
3'SL	HpaA (NLBH) adhesin	Clinical trial	Hp+ adults with dyspeptic symptoms	Oral administration, Results gained by Urea breath test	n/a	10-20g/day, split into 3 doses	Failed to cure or suppress Hp infection in humans	961
3'SL oligosaccharide from human and bovine milk	HpaA (NLBH) adhesin	*In vivo*	Rhesus monkeys	Oral administered treatment, observe biosies for clearance	7 clinical human isolates or naturally infected	3 daily doses of 3'SL 33mg/kg for 28 days; 167mg/kg with and without bismuth subsalicylate for 28 and 56 days respectively	3'SL = ~33% animals cleared, 3'SL + bismuth = 50% animals reduced colonisation	662
3'SL receptor analogue from bovine colostrum (BC); **3'SL-HSA, Glycoproteins**: fetuin, porcine gastric and submaxillary mucin, AGP (α-acid glycoprotein); **Sulfated glycoconjugates** (e.g.sulfatide)	HpaA (NLBH) adhesin	*In vitro*	Hu-Tu 80 cells (human duodenal adeno-carcinoma)	Inhibition & Removal assays from cell monolayers in microtiter plates	13 strains: clinical and standard strains including NCTC 11637	up to 10mg/ml for all inhibitors and ~20mg/ml for detachment using 3'SL with 3 x 10^7 Hp/ml	Inhibitiion of adhesion by 3'SL with most strains, not by 6'SL. All others mentioned inhibited adhesion Approx 40% removed with 3'SL. **NeuAc form of 3'SL responsible for inhibition (not NeuGc form)**	665

Table 11 continued

Inhibitor	Target adhesin /receptor	Study Type	Gastric Tissue model	Study Method	*H. pylori* strain	Effective concentration	Result & epitope responsible for inhibition	Reference
Target: HpaA (NLBH) adhesin								
Porcine mucin, heparin, fetuin, monosaccharides, KATO III membrane preparations, mAbs against Le b, sulfatide and GM_3	NLBH (HpaA)?? as blocked by fetuin; Hp-NAP?? as blocked by sulfatide mAbs	*In vitro*	KATO III cells (human gastric adenocarcinoma)	Inhibition-adhesion to suspended cells; analysed by flow cytometry	NCTC 11637	1mg/ml mucin, fetuin and membrane preps; 100U/ml heparin; 0.66mg/ml mAb	Mucin and heparin significamtly reduced adhesion, fetuin reduced adhesion, anti-sulfatide mAb marked reduction in adhesion, KATO III cell membranes inhibited (major component was sulfatide)	647
Fetuin (a sialoglycoprotein containing *N*-acetylneuraminyllactose) and **Neuraminidase (cleaves N-acetylneuraminyl-lactose**	NLBH (HpaA) adhesin	*In vitro*	Y-1 adrenal cells	Inhibition-adhesion to cell monolayers in microtiter plates;assessed by microscopy	ATCC CCL79	Fetuin: 2 or 4µg/ml Neuraminidase: 0.4U/ml. 1.3×10^8 Hp/ml	Monolayers pretreated with neuraminidase 47% cells had no adherent Hp. Fetuin blocked Hp adhesion to monolayer. **Epitope** is probably a **sialo-glycoprotein containing *N*-acetylneuraminyllactose**	963
Glycosphingolipids: **Sulfatide** and **gangliosides (GM_3; GM_1; GD_3** and **GD_{1a})**	Binds to NeuAc-NeuAc-Gal-Glc therefore possibly SabA adhesin or HpaA (NLBH) adhesin or polyglycosyl-ceramide (PGC)-binding adhesin.	*In vitro*	MKN-45 cells (human adenocarcinoma)	Removal and Inhibition-adhesion to cell monolayers in microtiter plates; results by ELISA reader	NCTC 11637	1×10^8 Hp/ml Inhibition: ≥125µg/ml. Removal: 500-1000µg	Inhibition adhesion: GD_3>GM_3>sulfatide. Removal: 500µg GD_3 (62%); 1000µg sulfatide (28%). Epitope: sialic acid	964
Target: PE-binding and Hp-NAP adhesins								
PE-coated lipobeads (phosphatidyl ethanolamine)	Possibly PE-binding adhesin (M-selectin; Lingwood et al., 1993)	*In vitro*	KATO III cells (human gastric adenocarcinoma) and tissue sections (normal human stomach)	Radiolabelling inhibition-adhesion assay (cell line) and fluoresecent inhibition-adhesion assay (tissue)	Clinical isolate	not mentioned	Significant reduction adhesion to KATO III cells and to tissue sections	965
Tween 20(detergent) and **Bismuth compounds**	Possibly PE binding adhesin (M-selectin) = PE and Gg4; or HpNAP = Gg4	*In vitro*	Immobilised lipid receptors (Gg_4 and PE) on microtiter plates	Microtiter plates coated with receptors Gg_4 and PE; assessed by ELISA plate reader	LC11 (see Lingwood et al.[667] for details)	Tween 20: 6µg/ml Bismuth salts: 10mg/ml	All bismuth salts inhibited adhesion to Gg_4 and PE. 100% inhibition achieved with bismuth subsialicylate (10mg/ml). Sulcrafate no effect.	966

Table 11 continued

Inhibitor	Target adhesin /receptor	Study Type	Gastric Tissue model	Study Method	*H. pylori* strain	Effective concentration	Result & epitope responsible for inhibition	Reference
Target: PE-binding and Hp-NAP adhesins								
BC (bovine colostrum) & **BCC** (bovine colostrum concentrate) from healthy cows; cholesterol, casein and lipoproteins removed)	Possibly PE-binding adhesin (M-selectin; Lingwood et al., 1993) = PE and Gg4 binding; or HpNAP = Gg4 binding	*In vitro*	Immobilised lipid receptors on TLC plates	Inhibition adhesion to phospholipid receptors PE and lyso-PE and glycolipids Gg_3, Gg_4, immobilised on TLC plates	Clinical isolate (child with DU and gastritis)	5-200mg/ml	BC 35-40% inhibition to Gg_4 and PE. 100% inhib to Gg_3 and lyso-PE; BCC 100% inhibition adhesion to all receptors. Gg_3, Gg_4, PE and lyso-PE are all Hp receptors	668
Sulfatides, sulfated gangliotetraosylceramide (Gg_4), galactosylceramide, N-AcNeuraminlactose, α-2-6- linked sialic acids	Possibly HpNAP = sulfatide and Gg4 binding or PE-binding adhesin = Gg4	*In vitro*	HeLa S3 cells (cervix carcinoma) and KATO III cells (human gastric carcinoma)	Inhibition-adhesion to cell monolayers in culture plates; assessed by microscopy	NCTC 11637 and 1 clinical isolate, 25.	50 - 100µg/ml	Inhibition of adhesion with Sulfatides, both ceramides and N-acetylneuraminlactose but not by α-2-6-linked sialic acids	657
Lactic acid bacteria strain JCM 1081 and TM105 (***L. reuteri***)	Possibly Hp-NAP = sulfatide binding	*In vitro*	Immobilised lipid receptors (GM_1 and sulfatide) on TLC plates	Inhibition of Hp adhesion to receptors asialo-GM_1 and sulfatide on TLC overlay assay; staining assessed by Image scanner and software	ATCC 43504	5 x 10^8 cells; inhibitor concentration unknown	100% inhibition of adhesion to both GM_1 and sulfatide	967
Target: HSP60 adhesin								
mAb (H20, IgM class) against **Hp-HSP60**	HSP60 adhesin	*In vitro*	MKN-45 cells (human gastric adenocarcinoma) and primary GECs from Hp- GC patient	Inhibition-adhesion to cells detected by flow cytometry	Clinical isolate (TK1029) from patient with GU and early stages of GC	5 x 10^8 Hp and 1 x 106 cell line. MKN-45 cells: 8, 40 and 200µg/ml Primary GECs: 125-1000µg/ml. High concs most effective	Pretreatment of Hp with mAb significantly inhibited adheision to both cell types. No inhibiton when MKN-45 cells pretreated with mAb.	674
pAb against ***Y. enterocolitica*-HSP60**	HSP60 adhesin	*In vitro*	MKN-45 cells (human gastric adenocarcinoma)	Inhibition-adhesion to cells detected by flow cytometry	NCTC 11638 and 12 x clinical isolates (from patients with Gastro-duodenal diseases)	Unknown	pAb inhibited adhesion to MKN-45 cells using 3 out of 13 Hp strains	675
Target: Other adhesins								
anti-Hp mAb A20 (IgM)	LPS adhesin	*In vitro*	MKN-45 and KATO III cells (human adenocarcinoma)	Inhibition-adhesion to cell monolayer detected by urease activity; inhibition-adhesion to cells detected by flow cytometry	NCTC 11638 and clinical isolate TK1029 (from GU patient)	500µg/ml mAb; 5 x 10^8 Hp and 1 x 10^6 cell line	Pretreatment of Hp with mAb significant inhibition of adhesion (74.4% to MKN45 cells, 27.5% to KATO III). Pretreatment of MKN45 cells did not inhibit adhesion.	968

Table 11 continued

Inhibitor	Target adhesin /receptor	Study Type	Gastric Tissue model	Study Method	*H. pylori* strain	Effective concentration	Result & epitope responsible for inhibition	Reference
Target: Other adhesins								
pAbs against **Hp-HSBP**	HSBP adhesin	*In vitro*	KATO III cells (human adenocarcinoma) and HeLa S3 cells (human epithelioid carcinoma)	Inhibition-adhesion to cell monolayers in microtiter plates; assessed by spectophotometry	ATCC 51932 and 51110; 12 clinical strains	Unknown	Between 32-87% inhibition of all strains to both cell lines	644
a-Hp yolk-derived Ab (IgY)	Possibly Urease	*In vivo*	♂ Hp+ mongolian gerbils and ♂Hp+ humans	Oral administration; assessed by Urea breath test (UBT), urease activity and some biopsies taken.	ATCC 43504	Gerbils: 20mg/day Humans: 10g/day Both given for 30days	Reduced Hp infection seen in all gerbils and some humans. May be due to **inhibition of urease activity** as NH_3 production was reduced by 94%	969
MFGM and **defatted MFGM** from bovine buttermilk; **LF** from bovine milk	Possibly sialic acid binding haemagglutinin (MFGM inhibited this adhesin Hirmo et al., 1998)	*In vitro* and *In vivo*	Hp+ BALB/cA mice and GECs from BALB/cA mice	*In vitro*adhesion-inhibition assay to monolayers in microtiter plates. *In vivo:* Oral administration, 10 days	EU317 clinical isolate passaged in mice (sialic-acid binding strain)	400mg/kg	All agents reduced adhesion to cell line and reduced gastric colonisation and gastritis *in vivo*	970
Sulphated exopolysaccharides from microalgae and **heparin**	Unknown	*In vitro*	HeLa S3 cells (cervix carcinoma)	Inhibition-adhesion to cell monolayers in microtiter plates; assessed by spectophotometry	?? Not mentioned	Heparin 10µg/ml, exopolysaccharides 0.4µg/ml; with 1×10^9 Hp/ml	Up to 50% inhibition with some exopolysaccharides. Sulphated exopolysaccharides inhibited adhesion better than heparin.	645
Lactic acid bacteria strain LG21 (***L. gasseri***)	Unknown	Clinical study	Hp+ adults	Oral administration of LG21 in yoghurt; assessed by levels of serum pepsinogen (PGI and II), urea breath test and examination of antral biopsies	n/a	90g of normal yoghurt consumed twice daily for 8 weeks followed by 8 weeks of same regimen using LG21-yoghurt.	26/29 patients reduced CO_2 (UBT) at 18 weeks showing reduced Hp colonies, changes in Raised PGI and II levels showed reduction in gastritis. Biopsies showed no. of Hp reduced 2 to 100-fold.	958
Lactic acid bacteria strain TM39 (***E. faecium***) from child faeces and **SCS** (supernatant from TM39 culture medium)	Unknown adhesin	*In vitro*	TSGH 9201 cells (human gastric carcinoma)	Inhibition-adhesion to cell monolayers in culture plates; assessed by microscopy	CCRC 17021	Equal concentration to Hp	TM39 and TM39-SCS gave significant reduction in adhesion to cell line	971
Lactic acid bacteria strain La1 ***L. acidophilus (johnsonii)***	Unknown	Clinical study	Hp+ adults	Oral administration of La1 culture supernatant; assessed by urea breath test and gastric biopsies	n/a	50 mls of whey-based La1 culture supernatant before meals and at bedtime for 14 days.	Lower UBT results in some patients. La1 supernatant had partial suppressive effect in humans	957

Table 11 continued

Inhibitor	Target adhesin /receptor	Study Type	Gastric Tissue model	Study Method	*H. pylori* strain	Effective concentration	Result & epitope responsible for inhibition	Reference
Target: Other adhesins								
M13 Phage expressing scFv against *H. pylori* antigens	Unknown	*In vivo*	Female BALB/c mice	Preincubation of Hp with phages then inoculated into mice; colonies in stomach grown and counted	NCTC 17874, 25, 66, 253 and 1139	Unknown	Reduced colonisation in mice (but maybe due to bactericidal effects)	972
Anti-ulcer agent **Ecabet sodium (ES)**	Unknown	*In vitro*	MKN-28 cells (human gastric adenocarcinoma)	Inhibition-adhesion to and **removal** from cell monolayers in microtiter plates; results by ELISA reader	10 x clinical isolates (from GU and Chronic gastritis patients)	1mg/ml; 1 x 10^9 Hp/ml	Pre-treatment of Hp with ES significantly reduced adhesion; pretreatment of MKN-28 with ES not affect adhesion; ES not remove bound Hp cells	973
Anti-ulcer agents **Rebamipide (R)** and **Ecabet sodium (ES)**	Unknown	*In vitro*	MKN-28 and MKN-45cells (human adenocarcinoma)	Inhibition-adhesion to cell monolayers in microtiter plates; results by ELISA reader	12 clinical strains	100µg/ml R, 1mg/ml ES.	Cells pretreated with R (50% inhibition) no effect with ES. Hp pretreated with R no effect, 50% inhibition with ES. Combination R+ES: pretreat cells with R and Hp with ES 100%inhibition. No effect the other way round.	974, 975
Whole human **milk** and milk **Ig's** from Hp-infected ♀	Unknown adhesin	*In vitro*	KATO III cells (human gastric adenocarcinoma)	Adhesion-inhibition assay; assessed by flow cytometry	Clinical isolates (antrum of children)	50% diluted	Whole Milk, casein and non-casein fractions but not Ig component inhibited Hp adhesion to KATO III cells. Non-Ig fraction responsible.	976
Buttermilk glycopoly-peptides	Unknown adhesin NOT sialic acid	*In vitro*	Porcine gastric mucin	**Removal** of bound Hp from mucin in 96-well plates	ATCC 43505, 43579; 5 clinical isolates	10mg/ml for 1-4 x 10^4 Hp/ml	Sugar component 86 -100% bound cells removed	840
BIC (from Hp-immunised cows)	Unknown	*In vitro* and *In vivo* and clinical study	Tissue sections (normal human stomach); BALB/cA and FVB/N Le b transgenic mice; Hp+ patients	Inhibition of adhesion to tissue sections. Oral administration to mice and patients	*In vitro*: NCTC 11637. *In vivo*: 119/95p and Hp1.	*In vitro*: 1 1/10 dilututed BIC. *In vivo*:Mice, 10-50mg/ml; Humans, 4g/day;	*In vitro*: BIC gave 90% inhibition using 0.1mg/ml, 100% using 1mg/ml. *In vivo*: 66% mice cleared rest had reduced colonies. In humans, 2 out of 8 patients infection cleared; children none cleared.	959, 960
BLF (bovine lactoferrin; from fresh skimmed milk)	Unknown	*In vitro* and *In vivo*	Murine gastric epithelial cells and male BALB/c mice	Inhibition of adhesion assay (fluorescence of bacteria detected by fluoroscanner) and oral administration to mice	Clinical isolate	*In vivo*: Mice: 10-50mg/ml; *n vitro*: >0.31mg/ml	1/10th of colonies found in treated mice (*in vivo*); significant inhibition of adhesion to GECs using >0.31mg/ml. **Possibly oligomannoside-type glycans of bLF involved**	977

Table 11 coninued

Inhibitor	Target adhesin /receptor	Study Type	Gastric Tissue model	Study Method	*H. pylori* strain	Effective concentration	Result & epitope responsible for inhibition	Reference
Target: Other adhesins								
Glycosylated glycoproteins and N-acetylneuraminyllactose	Unknown adhesin or perhaps Laminin (receptor)	*In vitro*	Immobilised laminin	Hp adhesion to radiolabelled laminin	NCTC 17874	25-100µg/ml; 1 x 10^8 Hp/ml	Glycosylated glycoproteins (up to 50% inhibiiton); N-acetylneuraminyllactose (70% inhibition). **Terminal sialic acids of laminin with sugar-binding proteins of Hp**	642
Bismuth subcitrate	Unknown	*In vitro*	Hep2, HM02 and primary cultured cells (all are human gastric carcinoma cells)	Inhibition of adhesion to cells determined by microscopic examination	7 x clinical isolates from gastritis and DU patients	100µg/ml; 1 x 10^7 Hp/ml and 0.5 - 1 x 10^5 ECs/ml	76% inhibition to HM02 cells and similar inhibition with the other cell types	978
mAbs against H-type 2, Le a and **Le b antigens**; combinations of the mAbs; **61kDa Hp outer membrane protein** (OMP)	BabA adhesin?? Le b receptor	*In vitro*	KATO III cells (human gastric adenocarcinoma)	Inhibition of adhesion to cells; assessed by flow cytometry	NCTC 11637	2.5 x 10^5 Hp/ml	Inhibition: Greatest inhibition = Le b+H type 2; Le b > inhibition Le a; H type 2 > inhibition Le b. All gave 10% or less inhibition. OMP = 64-74%. **H type 2 structure** important **receptor** for the **61kDa Hp adhesin**	979
K-casein from human milk	BabA adhesin?? Le b receptor	*In vitro*	Human gastric tissue sections rom healthy adult	Inhibition-adhesion assay	P466	100 - 200µg/ml	Human K-casein = 100% inhibition; **Le b** (fucose-containing CHO in K-casein)	980
Plasminogen, vitronectin, fetuin and other sialic acid-rich glycoproteins	Unknown adhesin	*In vitro*	Immobilised vitronectin (Vit) and plasminogen (Plas)	Inhibition of Hp adhesion to radiolabelled vitronectin and plasminogen	NCTC 17874	100µg/ml; 1 x 10^9 Hp/ml	Inhibition to Plas +Vit respectively = 90% and 10% by plasminogen; 35% and 85% by vitronectin. Lactoferrin = 24% inhibition to Plas.	14

5.2 Materials and Methods

5.2.1 *Helicobacter pylori* isolates

H. pylori NCTC 11637 was used in the study and labelled with FITC. Growth conditions and labelling procedure are outlined in Chapter 2 (sections 2.1 and 2.2) and Chapter 3.

5.2.2 Stomach sections

H. pylori-negative biopsies of human stomach (from patients with gastritis) were kindly donated by Professor Dino Vaira, University of Bologna, Italy with the consent of the Ethics Committee, St Orsola Hospital, Bologna. Formalin-fixed stomach biopsies were used whose epithelial cells expressed the Le b antigen (Le b stomach). Lewis status was determined by Immunohistochemistry. Procedures and conditions are outlined in Chapter 2 (section 2.3) and Chapter 3.

5.2.3 Inhibitors

The following inhibitors were tested for their ability to block *H. pylori* adhesion to Le b stomach sections: carbohydrates, domain antibodies, minibodies and bovine colostrum. The types of inhibitors, their target adhesin and source are summarised in **Table 12.**

5.2.3.1 Carbohydrates

The carbohydrates used in this study were receptor analogues for the *H. pylori* BabA or SabA adhesin (i.e. Le b and sLe x respectively). Le b was tested in a glycoconjugate form, having been conjugated to either human serum albumin (HSA) or Poly-D-Lysine (PL). The synthesis of these glycoconjugates meant that the receptor was present as an oligosaccharide (many sugar molecules linked to a core HSA or PL molecule), rather than in its single monosaccharide form; the aim of which was to produce a more potent inhibitor.

5.2.3.2 Domain antibodies

Several domain antibodies (dAbs), raised against the BabA adhesin of the J99 strain of *H. pylori* were used in this study. Controls for dAbs consisted of HEL4/pDOM2. HEL4 is a dAb against irrelevant antigen pDOM2 is the dAb expression vector routinely used by Domantis Ltd.

5.2.3.3 Minibodies

Minibodies with an Fab region against BabA were used in this study. Minibodies were stored at –20°C in a solution of PBS with 1%BSA to protect the antibody. The exact concentration was undetermined, but they were diluted 1:1 in PBS before use in an adhesion-inhibition assay.

5.2.3.4 Bovine colostrum

Bovine colostrum (BC) from a cow immunised with *H. pylori* (kindly donated by Professor Lennart Hammerstrom, Karolinska Institute, Stockholm, Sweden) was also tested for its inhibitory effect on *H. pylori* adhesion. This was tested because BC preparations from both healthy and *H. pylori* immunised cows, have been previously shown to inhibit *H. pylori* adhesion *in vitro* and *in vivo*.[668,956,959]

INHIBITOR	TARGET ADHESIN	SOURCE
Le b-HSA (Hexasaccharide)	BabA	1*
Le b(tet)PL	BabA	2*
Le b(hex)PL	BabA	2*
Domain Abs (x 3)	BabA	3*
Minibodies (x 3)	BabA	4*
sLe x	SabA	2*
Bovine Colostrum	?	4*

Table 12: Inhibitors of *H. pylori* adhesion used in this study. Le b-HSA = the glycoconjugate Le b-human serum albumin; Le b(tet)PL = the glycoconjugate Le b (tetrasaccharide)-PolyDLysine; Le b(hex)PL = the glycoconjugate Le b (hexasaccharide)-PolyDLysine; sLe x = sialyl Lewis x. *1 = Isosep, Sweden; 2 = Carbohydrate Synthesis, UK; 3 = Domantis Ltd, UK; 4 = Karolinska Institute, Sweden.

5.2.3.5 Combinations of inhibitors

Combinations of inhibitors (targeting two different *H. pylori* adhesins, SabA and BabA) were tested for their ability to block adhesion of the organism to Le b stomach sections. This was done to determine whether combinations of inhibitors would be more successful at lower concentrations than when tested singly. The inhibitor targeting SabA was sLe x, which was tested in combination with the following successful BabA inhibitors: Le b-HSA, Le b(hex)PL, dAb 25 and BC. Experiments were performed using a range of concentrations of the inhibitors. Two tissue sections were used for each concentration of inhibitor and all experiments were performed three times.

5.2.4 Adhesion-inhibition assay

Details of the adhesion-inhibition assay are outlined in Chapter 2 (sections 2.4.1 and 2.4.2) and Chapter 3. Two tissue sections were used for each concentration of inhibitor tested. All experiments were performed three times except for Le b(tet)PL, which was performed once and dAb 9 which was performed twice, due to their limited supply.

5.2.5 Removal assay

The inhibitors that were most successful at blocking adhesion of *H. pylori* to Le b stomach sections were additionally tested for their ability to remove *H. pylori* once it had already bound to the epithelial surface (therapeutic treatment), using a removal assay. Details of the removal assay are outlined in Chapter 2 (section 2.4.3). Experiments were performed three times using a range of concentrations of the inhibitor; two tissue sections were used for each concentration.

5.2.6 Quantification of binding

Sections were observed using a Laser Scanning Confocal Microscope, digital images of the sections were captured and converted to .TIF files for processing. Two photographs showing adjacent areas of the tissue were taken for each tissue section. Using these digital images, the number of adherent *H. pylori* was quantified using the

method devised in this thesis (ROI method with standard area method of counting) using Metamorph image analysis software.[905] Details of the method are described in Chapter 3 and the Appendix.

5.2.7 Statistical analysis

Statistical analysis was performed on the data using the Mann-Whitney U-test for non-parametric data.

5.3 Results

5.3.1 Inhibition studies

5.3.1.1 Carbohydrates

Inhibition of *H. pylori* adhesion using Le b-HSA **(Figure 33)** gave a dose response up to 250µg/ml where maximum inhibition of adhesion (59.7%) was achieved. At concentrations above 250µg/ml, inhibition was less than the maximum. Le b(tet)PL **(Figure 34)** showed very little inhibition of *H. pylori* adhesion and maximum inhibition achieved was 30% using 10µg/ml. At concentrations higher than this, inhibition of adhesion was slightly reduced. However, when using the hexasaccharide [Le b(hex)PL] to inhibit *H. pylori* adhesion **(Figure 35),** the percentage inhibition achieved was much higher. Maximum inhibition was 92.4% using 250µg/ml and inhibition gave a dose response. Inhibition of adhesion at the optimal concentration of Le b(hex)PL (250µg/ml) was highly significantly different ($p<0.01$) from the control (0µg/ml).

Inhibition of adhesion using sLe x (**Figure 36)** also followed a dose response and maximum inhibition (41.0%) was achieved using 100µg/ml but the difference from the control (0µg/ml) was not significant ($p>0.05$). At concentrations above this, inhibition had reached a plateau and was not significantly different from the maximum ($p>0.05$).

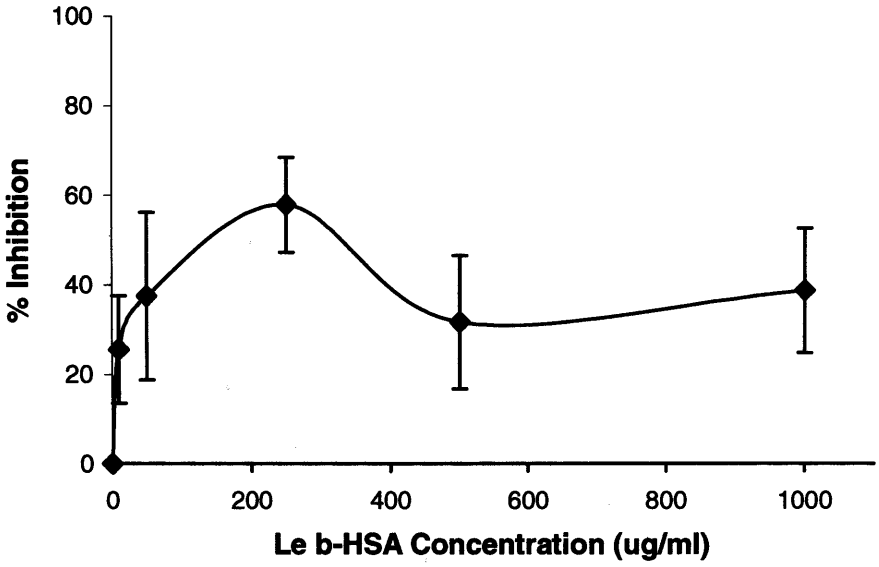

Figure 33: Inhibition of *H. pylori* adhesion to Le b stomach sections by Le b-HSA. The mean % inhibition of three experiments is shown with error bars.

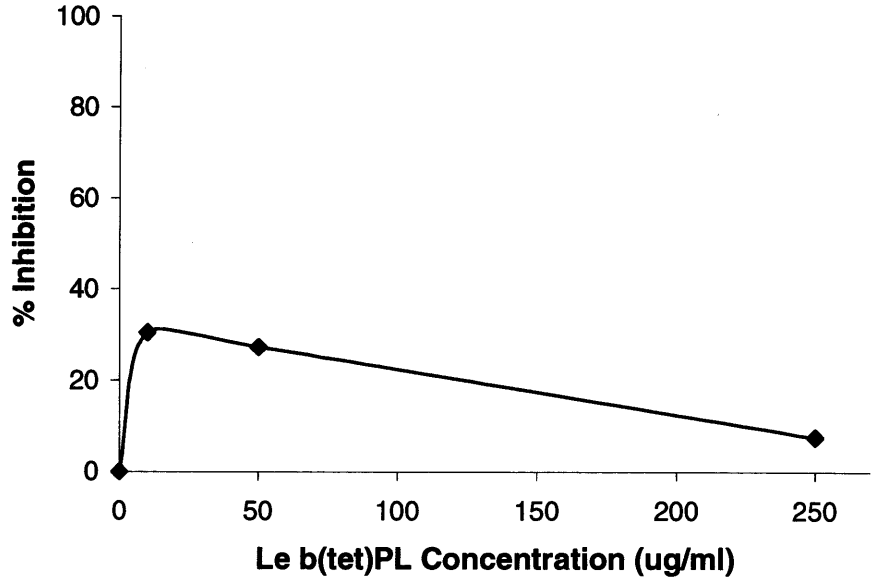

Figure 34: Inhibition of H. pylori adhesion to Le b stomach sections by Le b(tet)PL. The % inhibition of one experiment is shown.

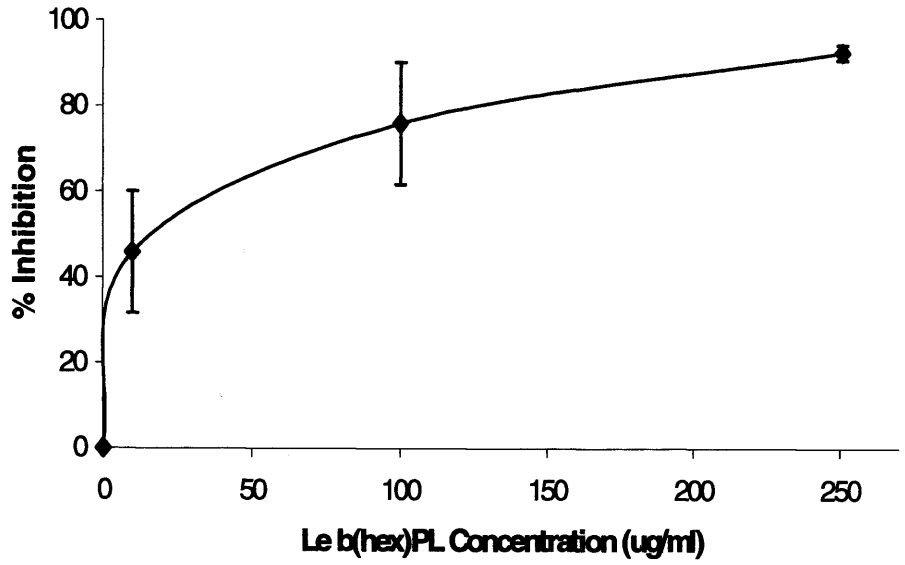

Figure 35: Inhibition of *H. pylori* adhesion to Le b stomach sections by Le b(hex)PL. The mean % inhibition of three experiments is shown with error bars.

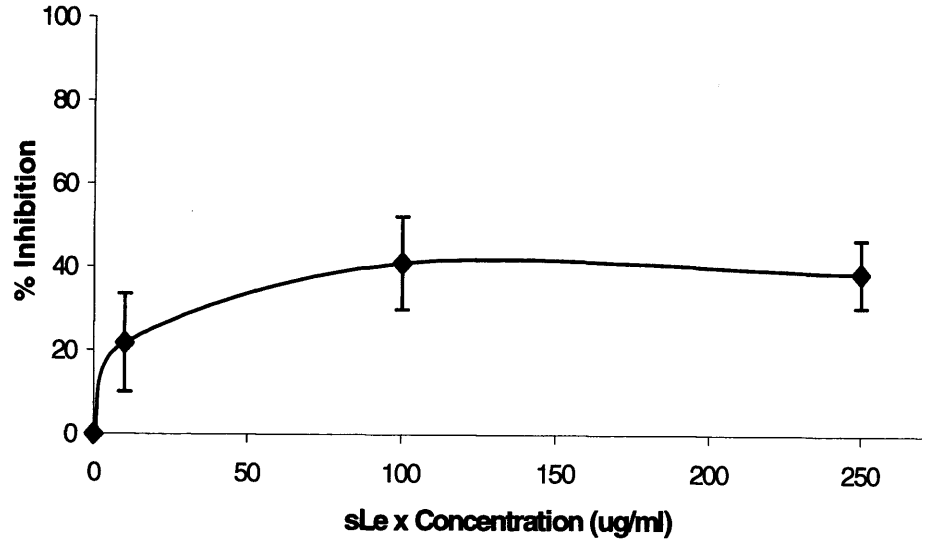

Figure 36: Inhibition of *H. pylori* adhesion to Le b stomach sections by sLe x. The mean % inhibition of three experiments is shown with error bars.

5.3.1.2 dAbs

The anti-BabA dAbs 9, 25 and 28 all inhibited adhesion of *H. pylori* to Le b stomach sections **(Figures 37 and 38)**. dAbs 9 and 28 were not effective since the percentage inhibition achieved by the control (HEL4/pDOM2) was approximately the same or higher than these two dAbs at all concentrations tested. dAb 25 also achieved lower percentage inhibition than HEL4/pDOM2 at concentrations of 10 and 50µg/ml. However, it out-performed the control at 100µg/ml and a maximum inhibition of 73.1% was achieved, which was significantly different ($p<0.05$) from the control (0µg/ml).

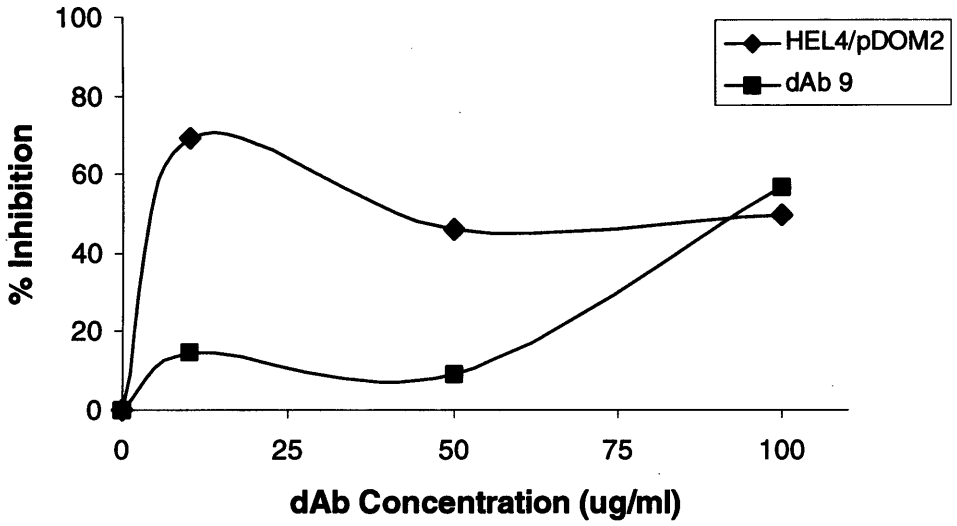

Figure 37: Inhibition of *H. pylori* adhesion to Le b stomach sections by anti-BabA dAb 9. The mean % inhibition of two experiments is shown.

5.3.1.3 Bovine colostrum

Bovine colostrum inhibited adhesion of *H. pylori* to Le b stomach sections **(Figure 39)** and maximum inhibition (60.8%) was achieved using 1000µg/ml [the difference was significant ($p<0.05$) from the control (0µg/ml)]. At lower concentrations the percentage inhibition fluctuated, but the differences were not significant ($p>0.05$) from the maximum.

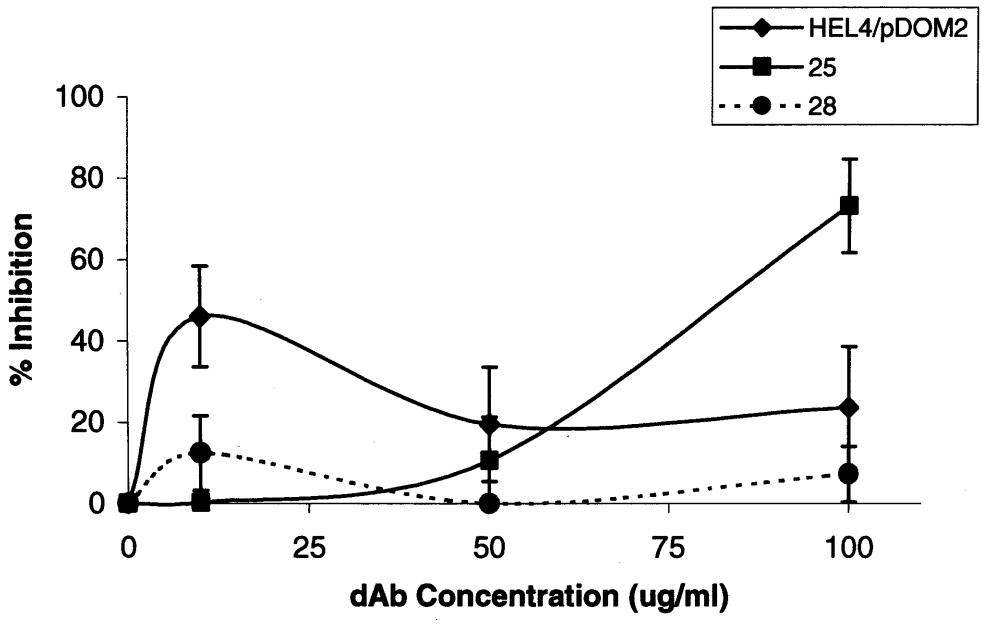

Figure 38: Inhibition of *H. pylori* adhesion to Le b stomach sections by anti-BabA dAbs 25 and 28. The mean % inhibition of three experiments is shown with error bars.

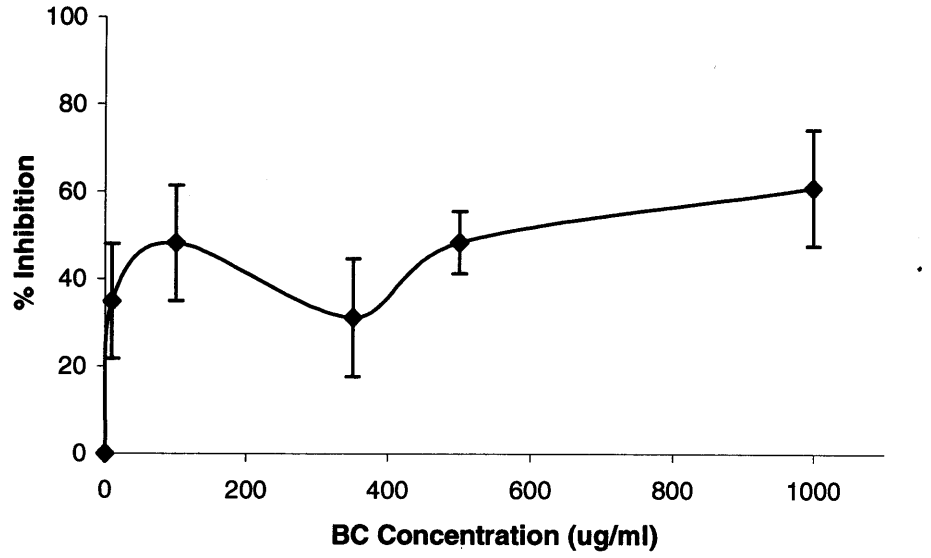

Figure 39: Inhibition of *H. pylori* adhesion to Le b stomach sections by bovine colostrum (BC). The mean % inhibition of three experiments is shown with error bars.

5.3.1.4 Minibodies

The minibodies inhibited adhesion to Le b stomach sections **(Figure 40)** by 23.8%, 52.5% and 45.5% (Mb 12, 19 and 30 respectively).

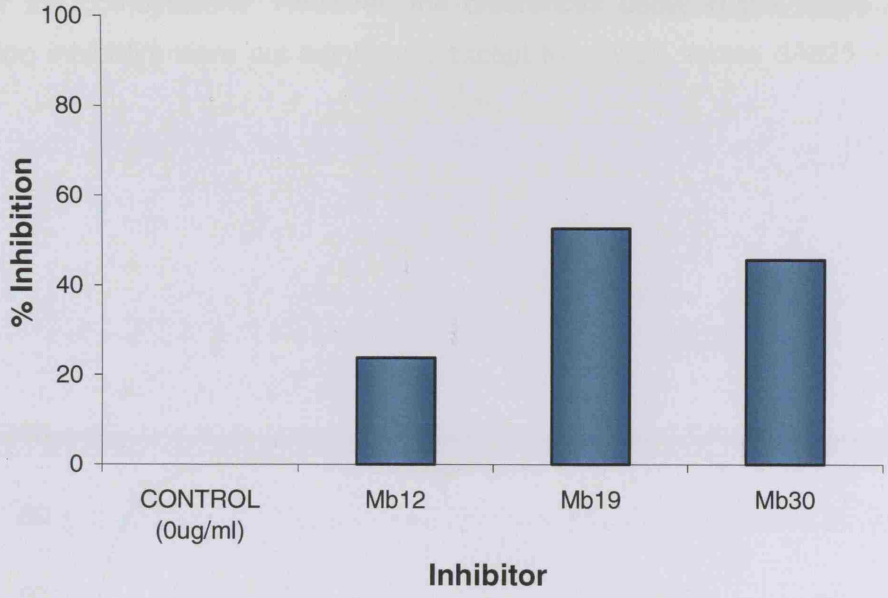

Figure 40: Inhibition of *H. pylori* adhesion to Le b stomach sections by minibodies (Mbs). The mean % inhibition of one experiment is shown.

5.3.2 Combinations of inhibitors

Adhesion of *H. pylori* was inhibited using combinations of the most successful inhibitors previously tested. The combination of sLe x and Le b-HSA was efficient from 10µg/mg **(Figure 41),** sLe x and dAb 25 from 50µg/ml **(Figure 42)** and sLe x and Le b(hex)PL from 10µg/ml **(Figure 43)**. With sLe x and Le b-HSA a maximum inhibition of 93.4% was achieved at 100µg/ml [the difference from the control (0µg/ml), was highly significant ($p<0.01$)]. sLe x and dAb 25 gave a maximum inhibition of 66.3% at 100µg/ml and there was a significant difference ($p<0.05$) between the maximum inhibition and the control (0µg/ml). Maximum inhibition achieved by sLe x and Le b(hex)PL was 95.9% at 100µg/ml [the difference from the control (0µg/ml), was highly significant ($p<0.01$)]. sLe x and BC in combination followed the same pattern of

inhibition as BC on its own (**Figure 44).** Inhibition fluctuated at all concentrations (but there was no significant difference between them, $p>0.05$) and the maximum inhibition was achieved at 100µg/ml (49.6%).

The combinations of inhibitors therefore out-performed the same inhibitors when tested singly, except for BC. Concentrations required for maximal inhibition were lower than the single inhibitors and the maximum percentage inhibition was always higher for the combinations. However, the differences between the single versus combination inhibitors were not significant, except for dAb25 versus dAb25 + sLe x ($p<0.05$).

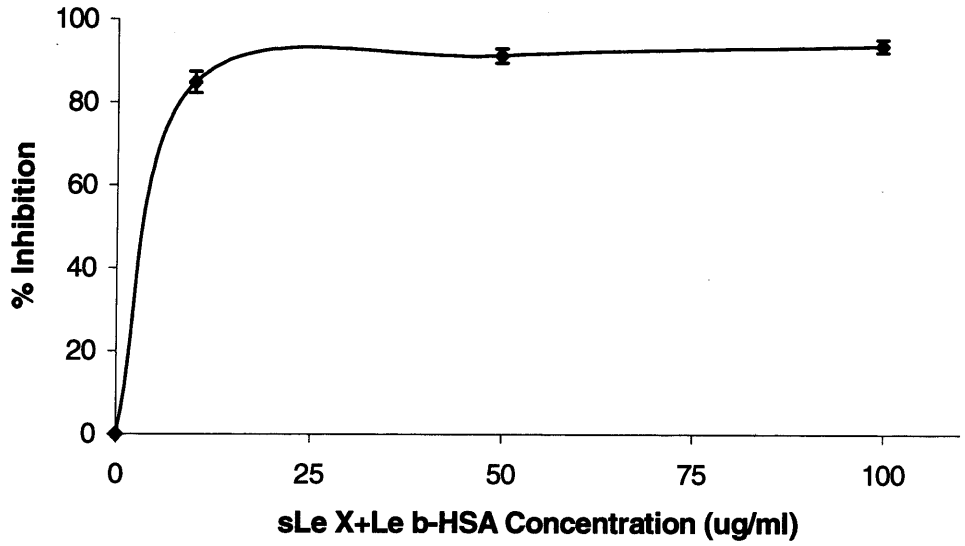

Figure 41: Inhibition of *H. pylori* adhesion to Le b stomach sections by a combination of sLe x and Le b-HSA. The mean % inhibition of three experiments is shown with error bars.

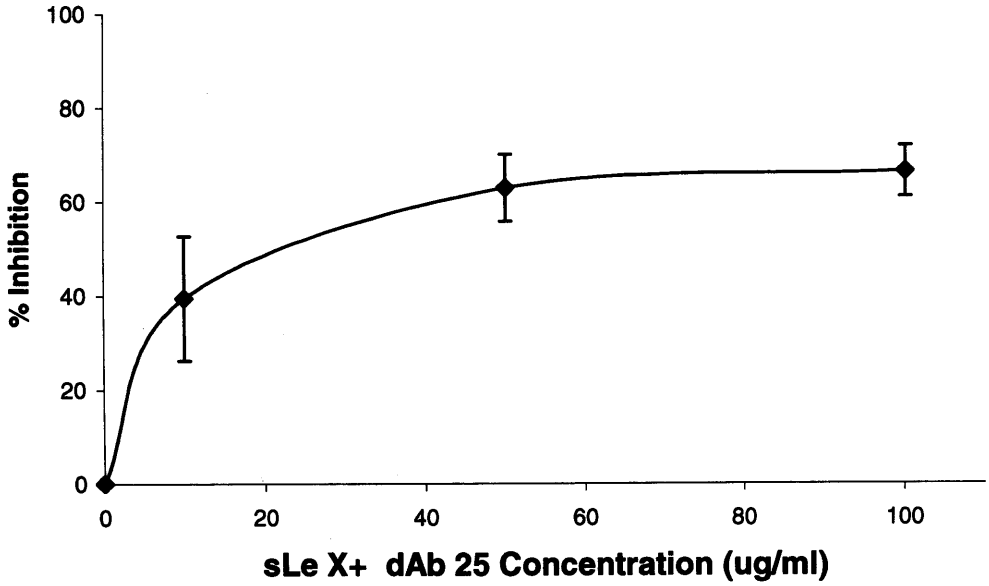

Figure 42: Inhibition of *H. pylori* adhesion to Le b stomach sections by a combination of sLe x and dAb 25. The mean % inhibition of three experiments is shown with error bars.

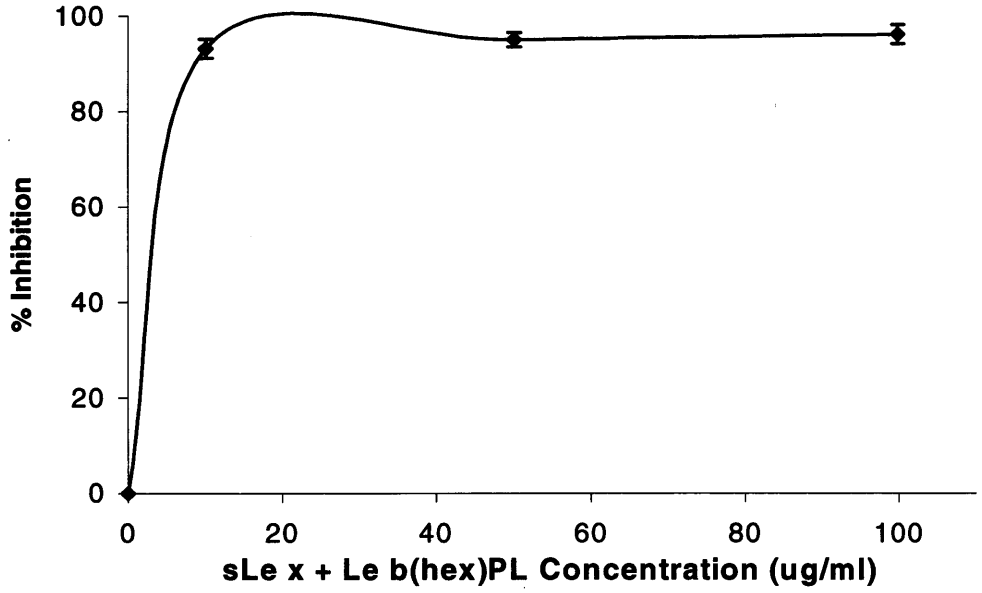

Figure 43: Inhibition of *H. pylori* adhesion to Le b stomach sections by a combination of sLe x and Le b(hex)PL. The mean % inhibition of three experiments is shown with error bars.

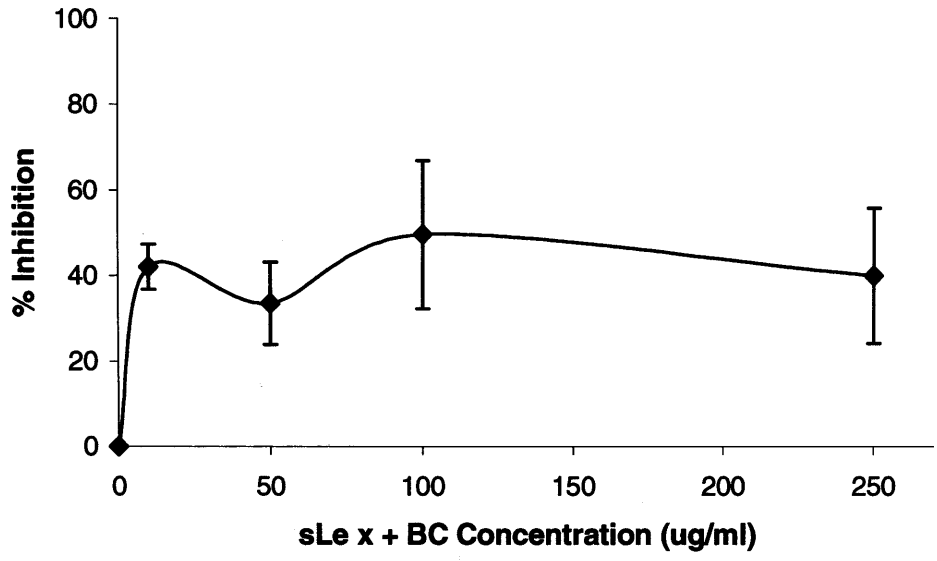

Figure 44: Inhibition of *H. pylori* adhesion to Le b stomach sections by a combination of sLe x and bovine colostrum (BC). The mean % inhibition of three experiments is shown with error bars.

5.3.3 Removal studies

Removal of bound *H. pylori* from the stomach epithelium with Le b-HSA (**Figure 45)** showed a dose response and the maximum number of adherent *H. pylori* cells were removed using 1000µg/ml (69.8% removed), which was a highly significant difference ($p<0.01$) from the control (0µg/ml). Using Le b(hex)PL (**Figure 46)**, maximum removal (71.5%) of bound *H. pylori* was achieved using 10µg/ml and the difference was not quite significant ($p = 0.064$) compared to the control (0µg/ml). The percentage of bound organisms removed was lower when using higher concentrations of Le b(hex)PL (44.7% and 69.2% at 100 and 250µg/ml respectively), however the difference was not significant ($p > 0.05$) compared to the maximum inhibition (10µg/ml).

The results of all the *H. pylori* inhibition and removal studies are summarised in **Table 13.**

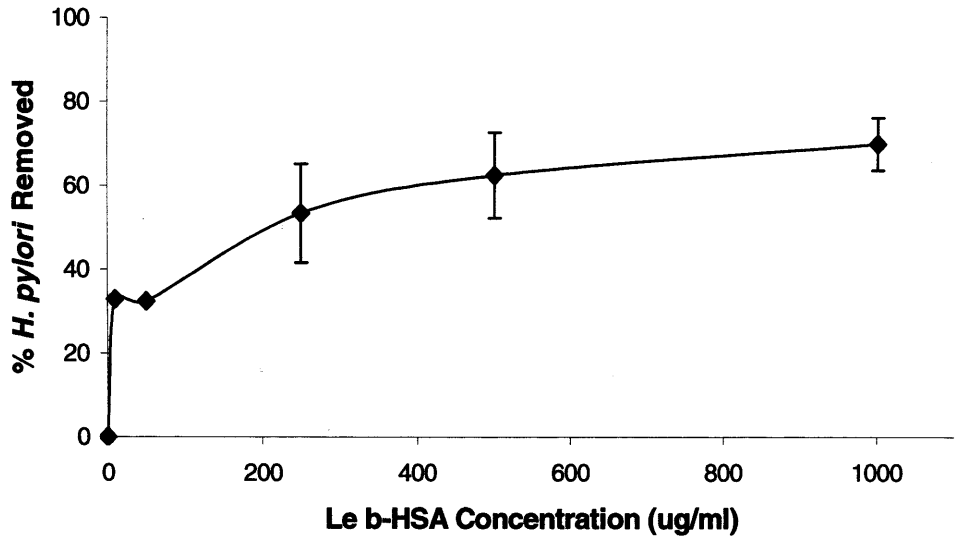

Figure 45: Removal of bound *H. pylori* from Le b stomach sections by Le b-HSA. The mean % inhibition of three experiments is shown with error bars.

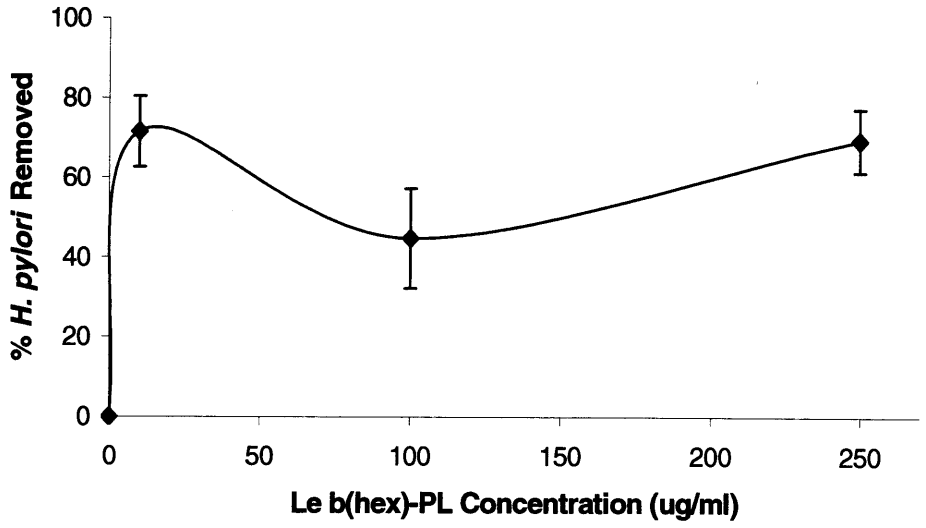

Figure 46: Removal of bound *H. pylori* from Le b stomach sections by Le b(hex)PL. The mean % inhibition of three experiments is shown with error bars.

INHIBITOR	TARGET ADHESIN	MAX. % INHIBITION	CONCENTRATION (µg/ml)
SINGLE INHIBITORS			
Le b-HSA	BabA	57.9	250
Le b(tet)PL	BabA	30	10
Le b(hex)PL	BabA	92.4	250
dAb 9	BabA	56.7	100
dAb 9 Control (HEL4/pDOM2)	?	64.7	100
dAb 25	BabA	73.1	100
dAb 28	BabA	12.4	10
dAb 25 Control (HEL4/pDOM2)	?	46	10
dAb 28 Control (HEL4/pDOM2)	?	23.6	100
Mb 12	BabA	23.8	?
Mb 19	BabA	52.5	?
Mb 30	BabA	45.5	?
sLe X	SabA	41	100
Bovine Colostrum	?	60.8	1000
COMBINATIONS OF INHIBITORS			
sLe x + Le b-HSA	SabA + BabA	93.4	100 (Plateau from 50µg/ml)
sLe x + Le b(hex)PL	SabA + BabA	95.9	100 (Plateau from 10µg/ml)
sLe x + dAb 25	SabA + BabA	66.3	100 (Plateau from 50µg/ml)
sLe x + BC	SabA + ?	49.6	100
REMOVAL EXPERIMENTS			
Le b-HSA	BabA	69.8	1000
Le b(hex)PL	BabA	71.5	10

Table 13: Summary of results of *H. pylori* inhibition and removal experiments.

5.4 Discussion

The first step of microbial infection is adhesion of the organism to the host tissue. By using molecules that mimic the adhesin or receptor, adhesion of *H. pylori* to gastric epithelial cells can be prevented, thus offering an alternative treatment to antibiotics for *H. pylori* infection. In this study, carbohydrates, dAbs and bovine colostrum (acting as receptor analogues) were found to inhibit the adhesion of *H. pylori* to human Le b+ stomach sections.

Le b-HSA, Le b(tet)PL and Le b(hex)PL all inhibited adhesion of *H. pylori* to Le b stomach sections. Le b(tet)PL was the least effective, probably because being a tetrasaccharide there were less molecules of Le b present (compared to Le b-HSA and Le b(hex)PL, which were both hexasaccharides), to inhibit *H. pylori* adhesins. The Le b hexasaccharide linked to PL was more effective than the Le b hexasaccharide linked to HSA. This may be because Le b(hex)PL contained more Le b molecules per carrier mol of PL (the exact number is unknown) than Le b-HSA (which contained 20mol of Le b per HSA mol). Le b(hex)PL would therefore be able to inhibit more BabA molecules using the same concentration of inhibitor. In fact, Simon et al.[665] showed that 3'SL was more effective as a multivalent glycoconjugate and suggested that this was due to enhanced avidity of the molecule, operating co-operatively among ligand-adhesin pairs interacting in tandem. Another possible explanation is that when conjugated to PL, the Le b molecules may have had greater steric flexibility than when bound to HSA and were thus able to bind with a greater affinity to the BabA adhesins of the *H. pylori* cells.

In the present study, a dose response was achieved using Le b(hex)PL, beginning to peak at about 250µg/ml, showing that the higher the concentration of inhibitor used, the more *H. pylori* adhesins were blocked. No dose response was obtained using Le b-HSA; maximum inhibition was achieved using 250µg/ml and at concentrations higher than this, inhibition was reduced. It may be that large amounts of glycoconjugate have an agglutinating effect on the bacteria and thus more bacteria bind to the epithelial surface and therefore inhibition is less at higher concentrations. In fact, more clumps of bacteria were seen on the epithelial surface of the tissue sections from the higher concentrations of Le b-HSA (500 and 1000µg/ml) compared to the control and lower concentrations of Le b-HSA.

Previous findings that Le b or Le b glycoconjugates inhibit adhesion of *H. pylori* to human gastric tissue sections and gastric epithelial cells[15,57,887] have been confirmed by this study. In particular, the studies of Borén et al.,[15] who also used Le b-HSA and found it to be a successful inhibitor of *H. pylori* adhesion to tissue sections. The present study however, required much higher concentrations of Le b-HSA to achieve a lower percentage of inhibition than that achieved in the literature. This is most likely to be because the strain used in the present study was different to that used by other groups and the number and exact form of BabA adhesin molecules expressed by each strain may be different. Also, the number of bacteria used in the present study appears to be higher than that of other studies, and thus it would be expected that larger amounts of inhibitor be required to inhibit adhesion. Additionally, counting of adherent cells by Borén's group[15] was always performed microscopically (manually by eye) and Reinhard's group[887] used NIH-Image image analysis software; both methods of which have been shown to be less accurate than counting with the aid of Metamorph image analysis software, which was used in this study (O'Mahony et al.[905] and Chapter 3).

Using sLe x to inhibit adhesion resulted in a dose response, indicating that the number of adhesins blocked increased with higher concentrations of the inhibitor sLe x. The maximum inhibition achieved using sLe x was lower than the Le b-hexasaccharide glycoconjugates. This was probably because there were less sLe x receptors than Le b receptors (BabA) present on the epithelial surface of the tissue sections, since the tissues were from *H. pylori*-negative patients and *H. pylori* infection increases the expression of sLe x.[57,821,981] However, sLe x would be present because the sections were from tissues with inflammation, which is also known to increase the expression of sLe x.[621,982,983] It may also be because SabA (which sLe x binds to), is not such a major adhesin as BabA; it has been suggested by Mahdavi et al.,[921] that SabA is a secondary adhesin. Apart from sLe x, SabA also binds to sLe a[621] and it is likely therefore in this study, that the binding of *H. pylori* to sLe x but not to sLe a was blocked by the soluble sLe x incubated with the bacteria in the experiments. Inhibition of adhesion by sLe x confirms previous findings in the literature where sialylated acid molecules (in particular 3'SL and sLe x) and glycoconjugates have been shown to inhibit adhesion of *H. pylori* to human gastric tissue sections, gastric epithelial cells and in rhesus monkeys.[57,621,647,657,659,662,665,956,964,984] In Mahdavi's study,[621] Inhibition using sLe x[621] was higher than that found in the present study but different strains were used and the tissue in Mahdavi's study was both *H. pylori*-positive and inflamed, and thus likely to express a larger number of sLe x molecules.

The anti-BabA dAbs 9, 25 and 28 all inhibited adhesion of *H. pylori* to Le b stomach sections. dAb 9 was less effective than the control HEL4/pDOM2 at the lower concentrations and only when 100µg/ml was used did it inhibit adhesion more than the control, but the percentage inhibition was not very different. dAb 28 was also less effective than the control at all concentrations tested. This would suggest that the binding regions of both dAbs 9 and 28 were not very specific for BabA. dAb 25 however, outperformed the control at 100µg/ml, inhibiting 73.1% of *H. pylori* compared to 23.6% with the control. This suggests that the sequence coding the binding region of dAb25 was much more specific for BabA than that of dAbs 9 and 28 and also suggests that at lower concentrations of dAb, non-specific inhibition of *H. pylori* adhesion occurs via a part of the dAb that is not the specific binding region (since the control HEL4/pDOM2, a dAb against an irrelevant antigen, also inhibited adhesion at low concentrations). At higher concentrations of dAb 25, inhibition of *H. pylori* adhesion was mediated to a greater extent by specific inhibition of the dAb interfering with the adhesin-receptor interaction.

The minibodies inhibited adhesion of *H. pylori* to the Le b tissue sections but were not quite as successful as the best dAbs and carbohydrates, although Mb19 gave a similar percentage inhibition to Le b-HSA. Concentrations of the Mbs were unknown and it may be that the concentration of Mb used was less than that of the other inhibitors, thus suggesting that less *H. pylori* adhesins were blocked, or maybe the binding regions of the Mbs were not as specific for BabA as the dAbs and carbohydrates were. However, because no irrelevant Mbs were available to test alongside the anti-BabA Mbs, it cannot be concluded for certain that the inhibition achieved was due to specific blocking of the adhesin-receptor interaction by the Mb. Only a handful of studies (see Table 1) have attempted to inhibit adhesion of *H. pylori* using antibodies and most of these did not target the BabA adhesin. The use of domain antibodies and minibodies are novel applications.

By pre-incubating *H. pylori* with bovine colostrum (BC), adhesion of *H. pylori* to Le b tissue sections was inhibited, but BC was less effective than the dAbs or the Le b (hexasaccharide) glycoconjugates, requiring 1000µg/ml for a lower maximum inhibition (60.8%) to be achieved. Inhibition of binding by BC occurs because BC has been shown to contain carbohydrates and antibodies that can block *H. pylori* adhesins.[665,959,960] However, because BC is not a pure preparation, larger amounts are required to achieve the same quantity of such inhibitors as pure inhibitor preparations would contain. This has been shown by the studies of Bitzan et al.[668] who found that a

concentrate of BC inhibited adhesion of *H. pylori* to immobilised *H. pylori* lipid receptors by a greater amount than the native BC did.

Several studies have investigated the specific components of BC that are responsible for inhibition of *H. pylori* adhesion. Bitzan et al.[668] found that BC inhibited the adhesion of *H. pylori* to the gastric lipid receptors PE (phosphatidylethanolamine) and the gangliosides gangliotriaosylceramide and gangliotetrasylceramide (Gg3 and Gg4 respectively) and this was shown in part to be mediated by colostral PE (phosphatidylethanolamine) or PE-derivatives rather than antibodies against *H. pylori* adhesins. BC is also known to contain large amounts of lactoferrin (bLF), which has also been shown to have anti-adhesive effects on *H. pylori* and oral administration of bLF was found to significantly reduce the colonisation rate of *H. pylori* in mice. This effect is thought to be due to the presence of glycans in bLF (which account for 11.2% of the molecular weight of bLF), rather than its iron-sequestering properties.[977] Simon et al.[665] extracted sialylated acids from BC and found that these were able to inhibit adhesion to human gastric cell lines. The present study used BC from *H. pylori* immunised cows, however with hindsight it would have been good to have tested preparations from non-immunised cows alongside this.

The most effective inhibitors found in this study (Le b-HSA, Le b(hex)PL, dAb 25 and BC) were each tested in combination with sLe x to determine whether this would increase their effectiveness at blocking *H. pylori* adhesion. It was found that using the glycoconjugates in combination with sLe x, resulted in more successful inhibition than when each inhibitor was used alone. The difference between the maximum inhibition achieved by Le b-HSA and Le b-HSA + sLe x and between Le b(hex)PL and Le b(hex)PL + sLe x, was not significant ($p<0.05$). This can be explained by the fact that the combinations of inhibitors targeted two different *H. pylori* adhesins and therefore were able to block more adhesins, thus requiring lower concentrations for more successful inhibition. Complete (100%) inhibition of adhesion was never achieved because additional adhesins to BabA and SabA are involved in the binding of *H. pylori* to the human stomach, for example, AlpA, AlpB and HopZ.[676,678,681,986] The presence of Le b(hex)PL resulted in greater inhibition than Le b-HSA when in combination with sLe x (although the difference was significant, $p<0.05$), as was also found when the inhibitors were tested alone. Again this is likely to be due to a greater number of Le b molecules present in the PL conjugate.

The combination of sLe x and dAb 25 was less successful than when using dAb 25 on its own, suggesting perhaps that some cross-reactivity occurred between sLe x and the dAb. The combination of BC and sLe x was found to be less effective than BC on its own (although the difference was not significant, $p>0.05$) which may indicate that a component of BC blocks or reacts with sLe x. It is possible that antibodies against *H. pylori* Le x are present in BC and may bind to sLe x (both of which share the same type of carbohydrate chain, Type 2), preventing it from binding to the SabA adhesin of *H. pylori* and thus inhibiting its adhesion to the stomach sections. This is plausible because the Le x antigen is expressed on LPS on the surface of *H. pylori* and is known to be a stimulant of the immune system[538] and therefore antibodies against Le x may be present in BC. Only one other study has investigated the effects of combinations of inhibitors on *H. pylori* adhesion.[974] They found that a combination of the inhibitors rebamipide and ecabet sodium, enhanced inhibition to approximately 100%, whereas inhibition using the inhibitors alone, was only 50%.

By incubating tissue sections (to which *H. pylori* had already bound) with Le b-HSA or Le b(hex)PL, bound *H. pylori* was removed from the tissue sections. A much higher concentration of Le b-HSA was required to remove less *H. pylori* cells from the epithelial surface than when Le b(hex)PL was used. Again, the effectiveness of the Le b(hex)PL is possibly due to there being a greater number of Le b molecules present per molecule of PL than on the HSA glycoconjugate. Interestingly, the percentages of *H. pylori* inhibited from adhering to the stomach sections was lower than the percentages of *H. pylori* removed, using the same inhibitors at the same concentrations. This may be due to the fact that different tissue sections (from different patients) were used and each section has a different number of *H. pylori* receptors present. It may also be that the inhibitors have an agglutinating effect on *H. pylori* when they are pre-incubated with the bacteria at high concentrations.

The ability of these inhibitors to remove bound *H. pylori* suggests that this strain of *H. pylori* has a higher affinity or attraction to the inhibitors than the receptors on the epithelial surface of the stomach sections and also suggests that they simply 'detach' from the epithelial surface molecules and bind to the molecules of inhibitor instead. This is probably because there is a higher density of receptor molecules present on each molecule of the inhibitor than there is on the surface of the stomach epithelium. By simple laws of chemistry (adhesion and intermolecular forces), the adhesins of *H. pylori* are more attracted to these soluble inhibitors than they are to the bound receptors and thus the bacteria are removed (detach) from the stomach surface. Only three studies in the literature have previously investigated and reported the ability of

an *H. pylori* anti-adhesin to detach bound bacteria from gastric epithelial cells.[665,840,964] The inhibitors in the present study were more effective than those in the literature (having taken into account the concentrations of inhibitor and bacteria used in the experiments), even though in two of the studies the same *H. pylori* strain was used as in this study. This may be because in the present study BabA was the target adhesin, which is thought to be a major adhesin of *H. pylori*.[15,621] The models representing gastric tissue also differed; those in the literature used cell monolayers or gastric mucin, which may contain different receptors, whereas tissue sections were employed in this study, which provides a better representation of the gastric environment.

To summarise, this study has shown that receptor analogues consisting of soluble glycoconjugates, dAbs, Mbs and BC are able to inhibit adhesion of *H. pylori* to human stomach sections, by blocking the specific interactions between the microbial adhesin and host cell receptor. This work is of importance for several reasons:

- Firstly, because several of the inhibitors are novel and have not been tested before, namely dAbs, Mbs and the glycoconjugate Le b(hex)PL; all of which were shown to be effective inhibitors of *H. pylori* adhesion.
- Secondly, the results of the study have shown that combinations of some inhibitors (i.e. targeting more than one *H. pylori* adhesin) were more effective than using single inhibitors against one adhesin. This is important for the therapeutic application of these inhibitors because a more effective treatment, which requires smaller amounts of inhibitor, will result in a reduction in the ultimate cost of the treatment.
- Thirdly, of significance is the finding in this study that Le b-HSA and Le b(hex)PL can not only inhibit adhesion of *H. pylori* to the gastric mucosa but are also able to remove bacterial cells once they have already bound. This strongly suggests that these two inhibitors in particular would make very potent candidates for both prophylactic and therapeutic treatment of *H. pylori* infection. This work is valuable because it indicates a possible therapeutic as well as prophylactic treatment for *H. pylori* infection, which is more relevant to the clinical situation, where the need is to treat patients once infection has already established. The potential mechanism by which adhesion-inhibitors work within the gastric environment, has been explained by Simon et al.[665] Since the stomach epithelial surface exfoliates every 48hrs, *H. pylori* generations must form new attachments to the epithelial lining to resist clearance caused by the flow of gastric contents. Therefore, molecules able to both inhibit adhesion as well as remove bound bacteria provide a very plausible therapeutic treatment.

- Fourthly, because soluble receptor analogues are specific for the microorganism being targeted, they have the advantage over antibiotics because they can inhibit the attachment of or remove the target bacterium without interfering with the native microflora.
- Fifthly, since this study has shown that multivalent glycoconjugates are more effective inhibitors of adhesion, future studies in which such glycoconjugates are bound to the surface of minute particles such as gold nanoparticles, may further increase the efficacy of an anti-adhesin treatment. By increasing the density of the inhibitors over a very small surface area they may prove to be an even more potent agent for both inhibiting and removing *H. pylori* cells.
- Finally, the method used to test the inhibitors in this study was shown to be a good indicator of inhibitors that are also effective *in vivo* (for *C. albicans*; see chapter 4) and therefore is a useful predictor of potentially successful therapeutic candidates. The successful agents found in this study will now need to be tested *in vivo* to confirm their efficacy.

Chapter 6

BACTERICIDAL AND ANTI-ADHESIVE PROPERTIES OF CULINARY AND MEDICINAL PLANTS AGAINST *H. PYLORI*

6.1 Introduction

Resistance to antibiotics is not limited to *H. pylori* and has been an increasing problem for many years. There is therefore a constant need for new antimicrobial agents and novel approaches to treatment, ideally preventing disease, such as inhibition of adhesion or vaccination.[159] Plants are known to be the source of phytochemicals which are beneficial for health and could also prevent diseases.[987] Among these phytochemicals, two are of particular interest in the case of infectious diseases: antimicrobial and anti-adhesive agents.

Numerous studies have been undertaken in order to find antimicrobial agents from plants against organisms ranging from viruses to protozoa.[831] The major concern is validation in humans with well-designed clinical trials and this has also been true for *H. pylori* infection. Several *in vitro* studies have looked at the effect of plant extracts on *H. pylori*. Antimicrobial effects have been reported for garlic,[988-990] green tea,[991] honey,[837] thyme,[992] berry extracts,[993] some Iranian plants[994] and the essential oils from several species of mint.[995] Some of these studies have been validated in animals and confirm the potential benefit of using plants as a source of antimicrobial agents against *H. pylori*. Although garlic and cinnamon have been tested in human clinical trials with no significant effect,[996] a recent study has shown that consumption of broccoli sprouts is associated with the eradication of *H. pylori* in some patients[997] but more work needs to be done determining the active ingredients of broccoli as well as performing studies on a larger number of patients.

The search for anti-adhesive agents represents the second alternative to antibiotic therapy, which has received less interest, although studies in animals and humans have proved its potential as a new therapy.[2] Successful inhibition of adhesion has been shown *in vitro* with cranberry juice against *H. pylori*[998] and for the seaweed *Cladosiphon fucoidan*.[999] Recently, Shibata *et al.*[1000] have demonstrated that

Cladosiphon fucoidan inhibits adhesion of *H. pylori* to porcine gastric mucin; by adding the plant to the drinking water of infected Mongolian gerbils, the prevalence of animals with infection was shown to be markedly reduced. Lengsfeld *et al.*[1001] have shown that by pre-incubating *H. pylori* with a fresh juice preparation of the fruit of the okra plant [*Abelmoschus esculentus* (L.) Moench], adhesion of *H. pylori* to human stomach sections was almost completely inhibited. Lengsfeld *et al.*[1002] have also demonstrated that acidic high molecular weight galactans from blackcurrant seeds could inhibit adhesion of *H. pylori* to human gastric mucosa tissue sections. Inhibition of *H. pylori* adhesion by polysaccharide fractions of *Panax ginseng* and *Artemisia capillaris* to a human gastric adenocarcinoma epithelial cell line has been shown by Lee *et al.* [1003]

As mentioned earlier, many plants have been shown to kill microorganisms but rarely have been studied for their anti-adhesive properties. The aim of this study was therefore to investigate both the bactericidal and anti-adhesive properties of 25 plants against *H. pylori*. Sixteen of them have never before been tested against *H. pylori* and are plants frequently used in cooking as well as in medicine in Sri Lanka, Iran and the Middle East. Moreover, the *in situ* adhesion assay optimised in this project has been used,[832] with stomach tissues expressing either the Lewis a (Le a) or Lewis b (Le b) antigen, in order to determine whether the plant extracts are inhibiting adhesion by blocking the major *H. pylori* adhesin BabA (which binds to Le b) or have an effect on other adhesins.

6.2 Materials and Methods

6.2.1 *Helicobacter pylori* isolates

H. pylori NCTC 11637 and six fresh clinical strains from patients with gastritis (kindly donated by Professor Dino Vaira, University of Bologna, Italy) were used in the study and labelled with FITC. Growth conditions and labelling procedure are outlined in Chapter 2 (sections 2.1 and 2.2) and Chapter 3.

6.2.2 Plants

Twenty-five plants were obtained from different sources and are described in **Table 14**. Extracts from the plants were made as follows: for turmeric, ginger, fenugreek, cumin, fennel, coriander and chilli, plants were boiled to 100°C in sterile distilled water (100mg/ml), filtered through sterile gauze, neutralised to pH 7.0 and then sterilised (extracts were kindly prepared and donated by Dr. Deepaka Weerasekera, Sri Lanka). Extracts from all the other plants were prepared as follows: plants were boiled in sterile distilled water (100mg/ml) for 10mins, allowed to cool and filtered through sterile filter paper (Grade 1, Whatman, UK). Fresh garlic, black peppercorns and cinnamon sticks were finely chopped before being boiled. All extracts were stored in the dark at -20°C until use. Before the experiments of adhesion, all 25 plant extracts were filtered again (Grade 1 filter paper, Whatman, UK).

6.2.3 Stomach sections

H. pylori-negative biopsies of human stomach were kindly donated by Professor Dino Vaira, University of Bologna, Italy with the consent of the Ethics Committee, St Orsola Hospital, Bologna. Two sets of formalin-fixed stomach biopsies were used: those whose epithelial cells expressed the Le a antigen (Le a stomach) and those expressing the Le b blood group antigen (Le b stomach). Lewis status was determined by Immunohistochemistry (as described previously in Chapter 2).

Five-micrometer sections of stomach were cut using a Leica SM2400 rocking microtome. Sections were collected on polished glass slides coated with Vectabond (Vector Laboratories, UK). Antigen-retrieval was used to expose the Le a and Le b antigens (for details of the method see Chapter 2 section 2.3.1 and Chapter 3 section 3.2.1).

English name	Sri Lankan name	Scientific name	Part of the plant used	Source	Use
Bengal quince	Belly	*Aegle marmelos*	Root	Sri Lanka	In cooking & medicine
Black Caraway (Europe)/Black Cumin (USA)		*Nigella sativa*	Seeds	UK, Supermarket	In cooking & medicine
Black pepper	Gammiris	*Piper nigrum*	Seed Berries/Fruit	UK, Supermarket	In cooking & medicine
Black tea	Thee	*Camellia sinensis*	Leaves and shoots	Sri Lanka	In cooking
Borage (Starflower)		*Borago officinalis*	Flowers	Iran	In cooking & medicine
Chilli	Mirise	*Capsicum anunum*	Fruit	Sri Lanka	In cooking & medicine
Cinnamon	Curundu	*Cinnamomum verum*	Bark	UK, Supermarket	In cooking & medicine
Columbo weed	Veneval	*Coscinium fenestratum*	climbing root	Sri Lanka	In medicine
Coriander	Kottamalli	*Coriandrum sativum*	Seeds	Sri Lanka	In cooking & medicine
Cumin (small cumin)	Suduru	*Cuminum cyminum*	Seeds	Sri Lanka	In cooking & medicine
Dill[a]	Sududuru	*Anthum graveolens*	Leaves	UK, Supermarket	In cooking & medicine
Fenugreek	Mathe seeds	*Trigonella foenum-graecum*	Seeds	Sri Lanka	In cooking & medicine
Garlic[a]	Sudulunu	*Allium sativum*	Bulb	UK, Supermarket	In cooking & medicine
Ginger	E'guru	*Zingiber officinale*	Rhizome	Sri Lanka	In cooking & medicine
Liquorice	Val'mee	*Glycyrrhiza glabra apofosa*	Stem	Sri Lanka	In cooking & medicine
Long Pepper	Tiphili	*Piper longum*	Seeds	Sri Lanka	In cooking & medicine
Nightshade	Ela battu	*Solanum surattense*	Fruit and root	Sri Lanka	In medicine
Nutmeg	Sadikka	*Myristica fragans*	Kernel	UK, Supermarket	In cooking & medicine
Oregano[a]	Oregano	*Origanum vulgare*	Leaves	UK, Supermarket	In cooking & medicine
Parsley[a]	Parsley	*Petroselinum crispum*	Leaves	UK, Supermarket	In cooking & medicine
Sage[a]	Minchi	*Salvia officinalis*	Leaves	UK, Supermarket	In cooking & medicine
Tarragon[a]	Tarragon	*Artemisia dracunculus*	Leaves	UK, Supermarket	In cooking & medicine
Threadstem carpetweed	Path paradagam	*Mollugo cerviana*	Seeds	Sri Lanka	In medicine
Turmeric	kaha	*Curcuma longa*	Rhizome	Sri Lanka	In cooking & medicine
Yellow-berried Nightshade	Katu val batu	*Solanum xanthocarpum*	Whole plant	Sri Lanka	In medicine

a: These plants were bought as both fresh and dried material

Table 14: Plants used in the study

6.2.4 Viable colony count

Overview of method

The most frequently used method to determine the bactericidal activity of various agents is to use 'viable counts' of bacteria. Suspensions of the organism (of a known concentration) are incubated with graded concentrations of the cidal agent under investigation. The negative control consists of bacteria without the addition of the cidal agent. At specific time points, 1ml is removed from each solution and decimally diluted. From each dilution, a further 100µl is removed and spread onto agar plates which are incubated for the time required for the organism to grow. After the incubation period, the number of colonies that have grown (viable colonies) are counted and the number of colony forming units, CFU per ml, are calculated and compared to that of the control. Studies of agents against *H. pylori* using this, or a similar method, have been frequently employed.[1004-1007]

Bactericidal activity of the plant extracts was determined by a viable colony count. One hundred microlitres of a suspension of 10^8 bacteria/ml was added to 900µl of plant extract for 60 mins. The control consisted of *H. pylori* incubated with sterile distilled water. Serial 10-fold dilutions were made and 100µl of each was plated at onto 5% horse blood agar (Oxoid, UK). These were incubated in gas jars under microaerophilic conditions for 3 days and colonies were counted (colony forming units per millilitre, CFU/ml). Plant extracts that completely inhibited *H. pylori* growth at 60 mins were further tested at 0, 15, 30 and 60 min intervals. All experiments were performed three times.

6.2.5 Microscopy

In order to validate the inhibition of adhesion by the plant extracts, any lytic effect of the extracts on the bacterial cells was first examined. FITC-labelled and non-labelled bacteria (1 x 10^8 cells/ml) were centrifuged (9000 rpm for 3 mins), re-suspended in 500µl of undiluted plant extract (100mg/ml) and incubated with continuous shaking for 1 hour at room temperature in the dark. After incubation, bacteria were centrifuged, washed in PBST and re-suspended in 500µl sterile distilled water. One drop of bacterial suspension was Gram-stained. Slides were observed for bacterial lysis using a Zeiss light microscope. All the plant extracts were examined, except long pepper and threadstem carpetweed, which were not tested due to their limited supply.

The negative control consisted of *H. pylori* incubated with sterile distilled water and the positive control of *H. pylori* incubated with Puregene PCR cell lysis solution (Flowgen, UK). The lysis experiments were performed twice.

6.2.6 Adhesion and adhesion-inhibition assay

Details of the adhesion-inhibition assay are outlined in Chapter 2 (sections 2.4.1 and 2.4.2) and Chapter 3.

All plant extracts were initially screened for inhibition of adhesion properties at a concentration of 50mg/ml (diluted in sterile distilled water) and incubated with *H. pylori* strain 11637 and Lewis b stomach sections. For those plants which had an anti-adhesive effect, the inhibition assay was repeated using Lewis a and Lewis b stomach sections and plants were diluted in sterile distilled water to concentrations of 5, 10 and 50mg/ml. Because 50mg/ml was found to give good inhibition of adhesion, for all further inhibition experiments, all plants were used at this concentration. Inhibition of adhesion experiments were then performed on three clinical strains. Controls consisted of bacteria re-suspended in sterile distilled water. Two tissue sections were used for each plant tested and all experiments were performed three times.

The plants that were most successful at inhibiting *H. pylori* adhesion were also tested as a combination for their anti-adhesive effects on *H. pylori* strain NCTC 11637 to Le b stomach sections. Concentrations of 10mg/ml and 25mg/ml of each plant were used and the experiment was performed twice.

6.2.7 Quantification of binding

Sections were observed using a Laser Scanning Confocal Microscope, digital images of the sections were captured and converted to .TIF files for processing. Two photographs showing adjacent areas of the tissue were taken for each tissue section. Using these digital images, the number of adherent *H. pylori* was quantified using the method devised in this thesis (ROI method with standard area method of counting; Chapter 3) using Metamorph image analysis software.[905]

6.3 Results

6.3.1 Bactericidal Properties of plants

Among the 25 plants tested for their bactericidal activity against *H. pylori*, eight showed no activity after 60 minutes of incubation: bengal quince, nightshade, garlic, dill, black pepper, coriander, fenugreek and tea (data not shown). The other 17 plants had activity (results are summarised in **Table 15**). Turmeric was the most efficient in killing the seven strains within 15 minutes. Ginger, cumin and chilli killed all strains within 30 minutes, whereas liquorice, oregano, black caraway and borage killed within 60 minutes. Cinnamon had a bactericidal activity against *H. pylori,* although the seven bacterial strains differed in their sensitivity. Moreover, post exposure, the bacterial colonies were very small, indicating some inhibitory effect on growth. Finally, the following plants showed a bactericidal activity against *H.pylori* but without achieving a complete inhibition of growth within 60 minutes: columbo weed, yellow-berried nightshade, long pepper, threadstem carpetweed, sage, tarragon, nutmeg and parsley. Small colonies were seen with yellow-berried nightshade, threadstem carpetweed and sage.

Table 15: Bactericidal activity of plants against *H. pylori*, determined using viable colony count. '% inhibition of growth' indicates the percentage of bacteria that were killed by the plants, (i.e. the colonies did not grow), compared to the control.

Plant name		NCTC strain	*H. pylori* clinical strains					
			1	2	3	4	5	6
Turmeric	% inhibtion of growth	100	100	100	100	100	100	100
	Minimum of time (min)	15	15	15	15	15	15	15
Ginger	% inhibtion of growth	100	100	100	100	100	100	100
	Minimum of time (min)	30	30	30	30	30	30	30
Fenugreek	% inhibtion of growth	0	0	0	0	0	0	0
	Minimum of time (min)	60	60	60	60	60	60	60
Cumin (small cumin)	% inhibtion of growth	100	100	100	100	100	100	100
	Minimum of time (min)	30	30	30	30	30	30	30
Coriander	% inhibtion of growth	0	0	0	0	0	0	0
	Minimum of time (min)	60	60	60	60	60	60	60
Chilli	% inhibtion of growth	100	100	100	100	100	100	100
	Minimum of time (min)	30	30	30	30	30	30	30
Bengal quince	% inhibtion of growth	0	0	0	0	0	0	0
	Minimum of time (min)	60	60	60	60	60	60	60
Columbo weed	% inhibtion of growth	99.9	99.9	99.9	99.5	99.8	99.9	99.8
	Minimum of time (min)	60	60	60	60	60	60	60
Yellow-berried Nightshade	% inhibtion of growth	99.9	99.7	99.5	99.9	99.9	99.9	99.9
	Minimum of time (min)	60	60	60	60	60	60	60
Liquorice	% inhibtion of growth	100	100	100	100	100	100	100
	Minimum of time (min)	60	60	60	60	60	60	30
Nightshade	% inhibtion of growth	0	0	0	0	0	0	0
	Minimum of time (min)	60	60	60	60	60	60	60

Table 15 continued

Long Pepper	% inhibtion of growth	99.9	99.9	100	99.3	99.7	99.9	99.9
	Minimum of time (min)	60	60	60	60	60	60	60
Threadstem carpetweed	% inhibtion of growth	99.9	99.7	99.9	99.9	99.9	99.9	99.9
	Minimum of time (min)	60	60	60	60	60	60	60
Garlic	% inhibtion of growth	0	0	0	0	0	0	0
	Minimum of time (min)	60	60	60	60	60	60	60
Parsley	% inhibtion of growth	99.6	99.3	99.9	99.6	99.9	99.99	99.8
	Minimum of time (min)	60	60	60	60	60	60	60
Dill	% inhibtion of growth	0	0	0	0	0	0	0
	Minimum of time (min)	60	60	60	60	60	60	60
Sage	% inhibtion of growth	99.9	99.9	99.7	99.5	99.9	99.9	99.9
	Minimum of time (min)	60	60	60	60	60	60	60
Oregano	% inhibtion of growth	100	100	100	100	100	100	100
	Minimum of time (min)	30	30	30	30	60	30	30
Tarragon	% inhibtion of growth	96.9	99.4	99.2	99.9	99.9	99.9	99.9
	Minimum of time (min)	60	60	60	60	60	60	60
Nutmeg	% inhibtion of growth	95.9	95.6	99.5	99.4	99.2	96.1	98.9
	Minimum of time (min)	60	60	60	60	60	60	60
Black pepper	% inhibtion of growth	0	0	0	0	0	0	0
	Minimum of time (min)	60	60	60	60	60	60	60
Cinnamon	% inhibtion of growth	100	100	100	100	99.9	99.9	100
	Minimum of time (min)	60	30	30	30	60	60	60
Black Caraway (Europe)/Black Cumin (USA)	% inhibtion of growth	100	100	100	100	100	100	100
	Minimum of time (min)	60	60	60	60	60	60	60
Borage (Starflower)	% inhibtion of growth	100	100	100	100	100	100	100
	Minimum of time (min)	60	60	60	60	60	60	60
Black tea	% inhibtion of growth	0	0	0	0	0	0	0
	Minimum of time (min)	60	60	60	60	60	60	60

6.3.2 Morphological studies

None of the 23 plant extracts tested caused lysis of *H. pylori* cells, as the organisms remained spiral and intact; identical to the negative control. Bacterial cells incubated with Puregene PCR cell lysis solution were lysed and no intact spiral shaped bacteria could be observed **(Figure 47).**

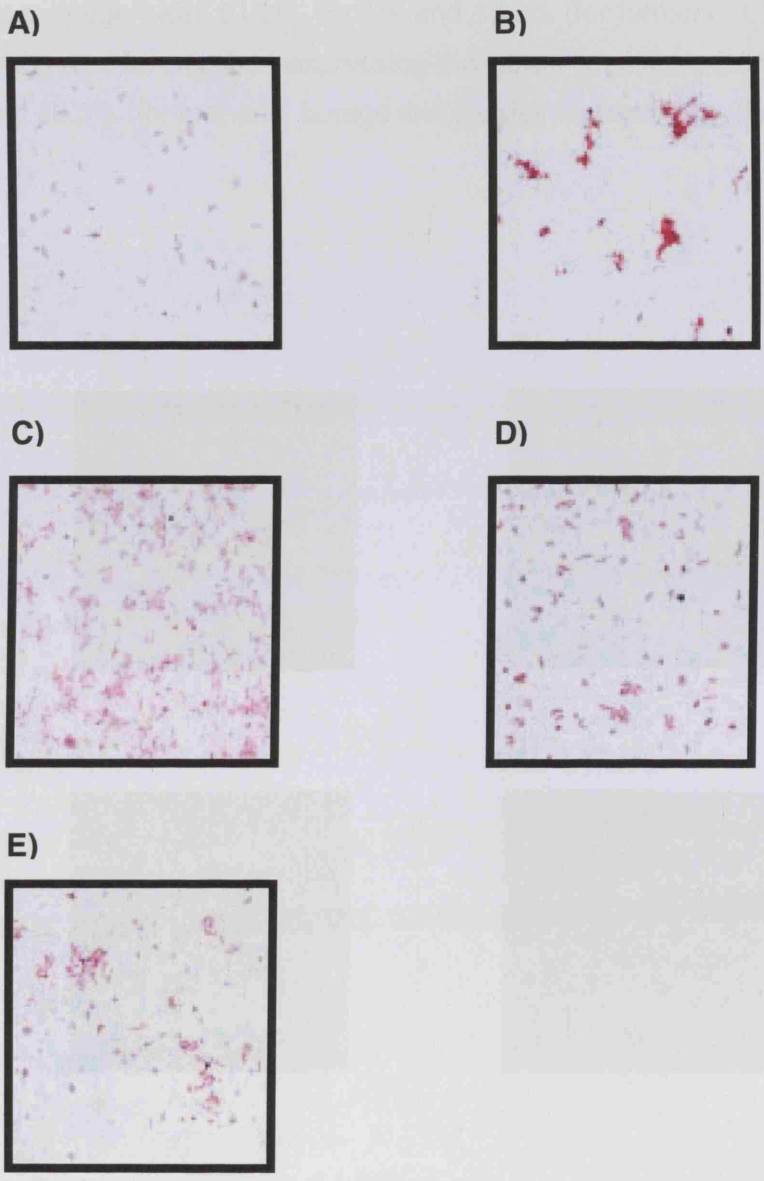

Figure 47. Bright-field images showing *H. pylori* lysis experiment. A) Negative control (*H. pylori* + distilled Water); B) Positive control (*H. pylori* + Cell Lysis solution); C) *H. pylori* + 0.05 g/ml turmeric; D) *H. pylori* + 0.05 g/ml borage; E) *H. pylori* + 0.05 g/ml parsley. Images taken using x100 objective.

6.3.3 Anti-adhesive properties of plants

Of the 23 plants screened for anti-adhesive properties, turmeric, borage and parsley (50mg/ml) were found to inhibit adhesion of *H. pylori* 11637 to stomach sections expressing the Lewis b antigen. **(Figure 48).** Further testing of these three plant extracts using *H. pylori* 11637 and 3 clinical isolates confirmed that they were able to inhibit *H. pylori* adhesion to stomach sections expressing either the Lewis b antigen or the Lewis a antigen. Mean inhibition of the four strains to stomach sections expressing the Lewis a antigen was 61.9%, 61.1% and 33.9% (for turmeric, borage and parsley respectively) and for sections expressing the Lewis b antigen, inhibition was 62.3%, 59.5% and 48.5% (for turmeric, borage and parsley respectively); **Figure 49**.

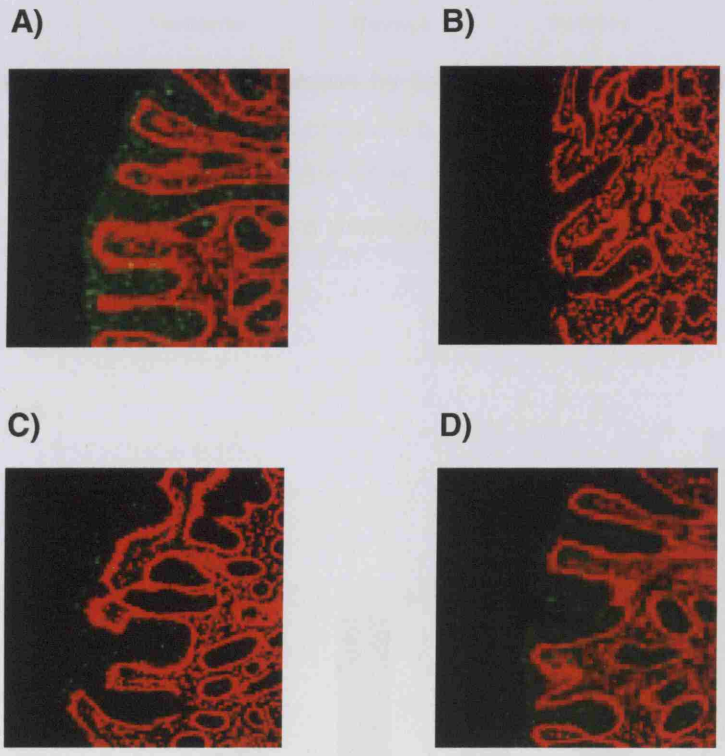

Figure 48. Confocal images showing inhibition of *H. pylori* adhesion by A) distilled water (control); B) turmeric (0.05 g/ml); C) borage (0.05 g/ml) and D) parsley (0.05 g/ml); to stomach sections expressing the Lewis b blood group antigen.

The combination of the three plant extracts inhibited adhesion of *H. pylori* 11637 to Le b stomach sections **(Figure 50)** by 78.3% and 96.4% (10mg/ml and 25mg/ml respectively). Inhibition of the plants on their own to Le b sections was 42.3%, 51.4% and 38.4% (for 50mg/ml turmeric, borage and parsley respectively).

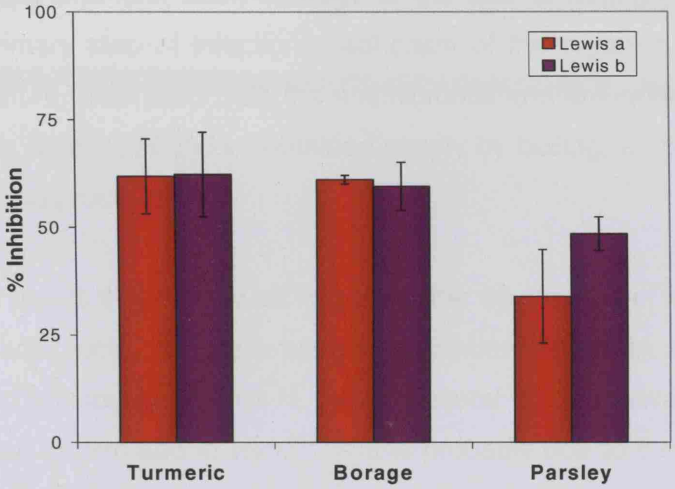

Figure 49. Inhibition of *H. pylori* adhesion by turmeric, borage and parsley to stomach sections expressing either the Lewis a or Lewis b blood group antigen. Experiments were performed three times using four strains of *H. pylori*. Mean percentage of inhibition is shown for each plant. Red bar = Lewis a stomach; Purple bar = Lewis b stomach.

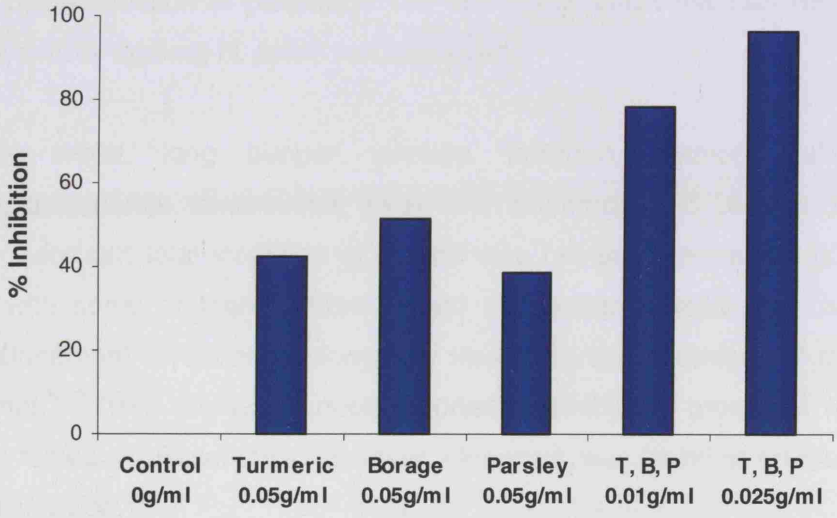

Figure 50: Inhibition of *H. pylori* 11637 adhesion to Le b stomach sections by the plant extracts turmeric, borage and parsley. Combinations of the extracts were also tested (represented by the abbreviation T, B, P) at lower concentrations. Experiments were performed twice using *H. pylori*. Strain NCTC 11637. Mean percentage of inhibition is shown for each plant.

6.4 Discussion

Investigations into plant materials as alternative sources of antimicrobials, has become more common over the past few years, due to the increased rate of development of antibiotic-resistant organisms. New strategies to combat infection are also being sought and one such strategy is the use of 'anti-adhesive' molecules, targeting the primary step of infection – adhesion of the organism to host tissue.[159] This study therefore, examined both the antimicrobial and anti-adhesive properties of 25 plant extracts against *H. pylori*, obtained simply by boiling, as would occur during the normal cooking process.

Of the eight plants that did not kill *H. pylori* after 60 minutes of incubation, (bengal quince, nightshade, garlic, dill, black pepper, coriander, fenugreek and black tea), two had been tested previously against *H. pylori*. Several studies have shown that garlic does kill *H. pylori in vitro* and *in vivo*.[988-990] It is probably due to the boiling method of extraction, that this effect was not observed in this study, since boiling has been shown to reduce the inhibitory activity of garlic against *H. pylori*.[1009,1010] Fenugreek sprouts have been shown to have high antimicrobial activity against *H. pylori*.[1011] In the present study, fenugreek appeared inactive but the seeds were examined rather than sprouts, which may account for the difference in results. Green tea catechins have previously been reported to have antibacterial effects against *H. pylori*, which was confirmed in Mongolian gerbils.[1012] Moreover, Chinese tea has been shown to reduce *H. pylori* infection in patients.[1013] In this study, black tea was tested but no bactericidal activity against *H. pylori* was observed.

Columbo weed, long pepper, parsley, tarragon, nutmeg, yellow-berried nightshade, threadstem carpetweed, sage and cinnamon had bactericidal activity against *H. pylori* but total inhibition of growth was not achieved within 60 minutes. Moreover, with some of them (yellow-berried nightshade, threadstem carpetweed, sage and cinnamon) a reduced colony size was observed. Nutmeg,[1014] tarragon[1015] and cinnamon[1016] have previously been reported to inhibit the growth of *H. pylori in vitro*. When tested in clinical trials, however, cinnamon was found to be ineffective at eradicating *H. pylori*.[1017]

Among the plants that kill *H. pylori*, turmeric was the most efficient, followed by cumin, ginger and chilli. These results confirmed what has been published before regarding turmeric,[1018] chilli[1019] and ginger,[1020] but cumin has not previously been tested. Other plants, never before been tested against *H. pylori* (borage, black

caraway and oregano), were also found to have a bactericidal effect. Finally, the previous reported bactericidal effect of liquorice has been confirmed.[1021,1022]

As well as looking at their bactericidal activity, all these plants were also tested for their anti-adhesive effects, using an *in situ* adhesion assay. Stomach tissues expressing either the Le a or Le b antigen were used. This was in order to determine whether the plant extracts inhibit adhesion by blocking the major *H. pylori* adhesin BabA, which binds to Le b, or have an effect on other adhesins. Most studies have not attempted to determine which adhesin is being blocked, and this may be important because combinations of plants targeting several different adhesins may provide a more potent therapeutic treatment.

Extracts of turmeric, borage and parsley were able to inhibit the adhesion of *H. pylori* strains to both Le a and Le b stomach sections. Turmeric was the most effective at inhibiting *H. pylori* adhesion to both Le a and Le b stomachs, followed by borage and parsley. From the results it seems that both turmeric and borage inhibit adhesins other than BabA, because the percentage of inhibition was almost exactly the same for Le a and Le b sections. Parsley possibly inhibits BabA as well as other adhesins, because inhibition occurred on Le a and Le b sections but was higher for Le b sections, indicating that it had a greater effect at inhibiting BabA. The morphological studies demonstrated that the reduction in adhesion caused by these plant extracts is due to true inhibition of adhesion and was not because the plants caused the bacteria to lyse. Tarragon is the only plant in this study that has been tested previously for its anti-adhesive effects, where it was found to inhibit *H. pylori* adhesion to human gastric adenocarcinoma cells.[1003] However, in the present study tarragon failed to inhibit *H. pylori* adhesion to human stomach sections. This discrepancy is probably due to differences between using cell-lines and whole tissue.

By combining the three plant extracts, inhibition of *H. pylori* adhesion (to Le b stomach sections) was even more effective than the single extracts on their own. Inhibition of adhesion using the combination was higher using lower concentrations. This is probably because the extracts were able to target and block several different adhesins simultaneously, thus lower concentrations were required to inhibit a higher number of bacterial cells.

Plants contain multiple organic components including phenols, quinones, flavones, tannins, terpenoids and alkaloids, all of which are known to have cidal effects on bacteria.[831] These substances are also water-soluble and therefore very likely to be

present in the plant extracts produced in this study and are likely candidates responsible for the killing effect of the extracts on *H. pylori*. Plants also contain many water-soluble proteins, lectins and carbohydrates which may bind specifically to sugar residues, polysaccharides, glycoproteins or glycolipids such as the adhesins present on the cell surface of *H. pylori*. In the event of such interactions occurring, the result would be to block the availability of the adhesin to its receptor and hence prevent adhesion of the bacterium to the stomach sections.

Despite the success of many plant materials at killing microorganisms, resistance can develop to these too. The present work is of importance because plant extracts were shown to inhibit adhesion of *H. pylori* to the stomach – an alternative strategy to bactericidal compounds. Because inhibition of adhesion works on the principle of sterically blocking microbes from attaching to host tissue, the likelihood of resistance developing in the microbe, using agents which kill is less likely.

Globally, *H. pylori* is the major cause of gastric cancer and has been classified as a Class I carcinogen by the WHO. Some plants are known to have anti-ulcerogenic and anti-cancer effects. Most of the plants tested here which have bactericidal and/or anti-adhesive properties, have also been shown to have anti-ulcerogenic or anti-cancer effects. These are: turmeric,[1023,1024] ginger,[1025,1026] cumin,[1027,1028] borage,[1029] liquorice[1030] and parsley.[1031] However, chilli has been shown recently to have detrimental effects on the gastric mucosa.[1032] The anti-cancer and anti-ulcerogenic effects of ginger, cumin, liquorice, parsley, turmeric and borage, combined with their cidal and anti-adhesive properties (as shown in this study), suggests that ingestion of these six plants could have important therapeutic implications for patients with *H. pylori*-induced peptic ulcer disease or gastric cancer. As turmeric kills 100% of organisms within 15 minutes, it could therefore be a useful anti-*Helicobacter* agent *in vivo*, because, if given orally, it would be able to kill *H. pylori* despite the short amount of time that it would remain in the stomach during digestion. Moreover, these plants could be used in combination with antibiotics, possibly increasing the success of eradication, as has been shown *in vitro* for cranberry juice.[1033]

Most studies use plant extracts that are obtained by chemical processing, for example, ethanol extracts. In developing countries where antibiotics are less freely available, such processing methods of plants would be impractical and/or expensive. The present study is therefore of importance because it has demonstrated that several plant extracts are effective against *H. pylori* and are obtained simply by boiling the plants. Such a method may provide a treatment that is simple, relatively

inexpensive and could be incorporated into the normal diet of the patient, which is highly favourable. Herbal treatments are usually unregulated and the safety of plants consumed is often unknown. The plants used and shown to be effective in this study are already commonly consumed and thus they are already known to be safe.

Although the cidal and anti-adhesive effects of ginger, cumin, liquorice, parsley, turmeric and borage have been shown *in vitro,* further studies have to be carried out investigating their effects *in vivo,* to see whether the extracts are able to remain effective despite the harsh process of digestion. Further analysis is also required to identify the exact components responsible for inhibition and anti-adhesion.

Chapter 7

ADHESIN-RECEPTOR INTERACTIONS INVOLVED IN ADHERENCE OF *HELICOBACTER PYLORI* TO DIFFERENT TOPOGRAPHICAL REGIONS OF THE HUMAN STOMACH

7.1 Introduction

There are two main topographical areas in which *H. pylori* infection occurs within the human stomach: the lower (antral) region or both the lower (antral) and upper (corpus and fundal) regions together. It is extremely unusual for the fundus alone to be infected. *H. pylori* initially colonises and is mainly confined to the gastric antrum[554] resulting in antral gastritis, but in some people the infection spreads to include the upper parts of the stomach such as the corpus and fundus,[555,1034] resulting in a pangastritis. When gastric function is normal, *H. pylori* remains confined to the antral surface, however, peptic ulceration may occur when the balance between host and bacteria is disturbed.[554] If left untreated, with time, *H. pylori* infection of the antrum spreads[1035] to where it either affects the duodenum (eventually causing duodenal ulcers due to increased acid production) or to further regions of the stomach, resulting in chronic gastritis. Two virtually exclusive disease pathways result from this topographical distribution; the duodenal ulcer pathway is the result of *H. pylori* infection of the antrum, whilst infection of both the antrum and fundus leads down the gastric cancer pathway. Only a small number of infected patients will eventually develop duodenal ulcer or gastric cancer.

Little is known about the colonisation and adhesion of *H. pylori* to different parts of the stomach and why in some people the bacterium is only able to colonise the antrum, while in other people it is able to spread to the fundus. It is known that secretion of acid and local acidity are regulators of *H. pylori* colonisation density in the stomach and it has been hypothesised that this may be the reason for chronic gastritis occurring in dissimilar topographical subtypes.[556,557]

Despite its ability to survive within an acidic environment, the distribution of *H. pylori* within the human stomach is strongly influenced by the presence of acid.[557] In the normal human stomach, the entire stomach surface contains mucous-secreting goblet cells, however, the distribution of the acid-secreting cells differs with

topographical region. The corpus and fundus of the stomach are lined with numerous parietal cells whose function is to secrete acid, whereas within the antrum, acid-secreting parietal cells are absent. In human infection, *H. pylori* primarily and preferentially colonises the antral region of the stomach. This is thought to be because the body of the stomach is the site of acid production and therefore the concentration of acid within this region is too high for the bacterium to neutralise effectively, whereas in the antrum where acid is not produced, the concentration of acid is low enough for the bacterium to deal with and here it is able to survive.

The role of acid in limiting the distribution of *H. pylori* and protecting the body of the stomach from infection is clearly shown by patients who have genetically-determined low levels of acid secretion (hyposecretors). In such people, antral *H. pylori* infection is able to spread to the body of the stomach where it results in a pangastritris. In hypersecretors (who produce excess acid), the high level of acid in the body of the stomach confines the organism to the antrum resulting in antral gastritis.[551] In some hyposecretors, antral infection and gastritis may eventually heal, resulting in hyperchlorhydria (low levels of acid) which leads to atrophic gastritis that is predominant in or limited to the corpus.[557] Dixon[1036] and Logan et al.[1037] have shown that *H. pylori* infection begins at the antrum and spreads to the corpus (where acid-secreting parietal cells are located) if acid secretion is reduced by therapy or infection-induced mucosal damage. Interestingly, there are very few reports of *H. pylori* infection or gastritis that is only limited to the fundus and the recommended sites for taking biopsies to detect *H. pylori* in both adults and children, are from the antrum and corpus.[1038,1039]

Apart from acid secretion there are other factors involved in determining the topographical location and clinical outcome of *H. pylori* infection. Host factors (including host genetics), environmental factors and microbial factors all have a role to play (see **Table 16**). Because even if acid concentration is optimal for bacterial survival, the strain must have the necessary virulence factors (such as the correct adhesins to attach to the stomach epithelium), to survive. It has also been implied that the preference of the organism for the antrum may be due to the presence of the necessary receptors (that enable *H. pylori* to bind to its target cells) in the antrum rather than the body/fundic regions.[558,559,1040,1041]

Several other possible explanations for differences in topographical location of *H. pylori* infection have been suggested and it is very unlikely that single factors alone are responsible, but rather a combination of them all. Based on the work of Allen et al.[726] and Amieva et al.[709] on intracellular *H. pylori*, Akada et al.[558] have suggested a

'compartment model' for *H. pylori* distribution. Factors affecting *H. pylori* distribution and the creation of suitable niches for colonisation are: differences in pH; nutrient availability; host defences such as distribution of inhibitors of bacterial growth (for example, reactive oxygen species, anti-bacterial peptides, lymphocytes and macrophages and so on); bacterial defences (such as acid-resistance, virulence genes, resistance to host defences) and expression of adhesins by the infecting strains and receptors present on the gastric epithelium.[558] Terry et al.[1042] have also shown that chemotaxis has a role in maintenance of infection and colonisation of different gastric regions. WT (chemotactic) strains of *H. pylori* were shown to colonise both the corpus and antral regions of the stomach of FVB/N mice, whereas non-chemotactic mutants were only found in the corpus.

One of the major factors that may be involved in determining topographical location and disease outcome (as mentioned earlier), is strain variation in the expression of different virulence factors. Indeed it has been shown that strains expressing certain virulence factors are more likely to cause certain diseases. Virulence factors that have been shown to be involved in *H. pylori*-related diseases are summarised in **Table 16.** Strains that are more virulent may well be able to colonise more difficult regions of the stomach such as the body/fundus, which is usually harder for *H. pylori* to colonise since this is the site of acid secretion. Needless to say, colonisation pattern and disease outcome cannot be attributed to a single virulence factor but rather to combinations of factors that either enhance or reduce the organism's ability to cause disease or be removed by the host. It has been shown that patients infected with strains of *H. pylori* that co-express certain virulence genes (such as *cagA* and *vacA*) are at the highest risk of developing the more serious diseases.[517,518,604-606,1043]

Cell surface adhesins are one specific type of virulence factor that *H. pylori* possesses and much strain variation exists in the specific adhesins that they express.[534,535,621,1053,1054] It may therefore be possible that the topographical differences of *H. pylori* colonisation are caused by differences in the expression of adhesins of the infecting strains. Expression of epithelial cell surface molecules may also vary with topographical location in the stomach and thus differences may exist in the adhesin-receptor interactions involved in bacterial colonisation to different regions of the stomach. Little work has been done to investigate this hypothesis and to determine whether different bacteria-host adhesin-receptor interactions are occurring in the different regions of the stomach and whether this accounts for the topographical differences seen.

Factor	Risk of Disease	References
Host Factors		
IL-1ß and IL-1RN genotype (High production)	Gastric Cancer / PUD	590, 603
HLA type	Possible role in Gastric Cancer	1044
Mucin type (small alleles at MUC1+6 loci)	Gastric Cancer	1045,1046
Environmental Factors		
Helminth infection	Reduced Cancer risk	1047
Smoking	Gastric Cancer	578, 1048
Diet (Low fruit + vegetables, high salt, Low Vitamin C, high nitrates/nitrites)	Gastric Cancer	596, 597, 1049, 1050
Poor childhood environment / living conditions	Increased Risk of Hp colonisation	1051
Microbial Factors		
Colonisation pattern:		
Antrum	Duodenal Ulcer Disease	551
Fundus	Gastric Ulcer / Gastric Cancer	551
Virulence Genes:		
cagA	Gastric Cancer / PUD	514, 603
vacA (s1a/m1)	Gastric Cancer / PUD	517, 602
vacA (s1b/m2)	MALT Lymphoma	517
iceA1	PUD	518, 520
oipA	PUD / Gastric Cancer	603
HP0169 (Collagenase)	Increased severity of ulcer	1052
Adhesins:		
BabA2	Higher risk of disease	604
sabB	Duodenal Ulcer Disease	679

Table 16: Factors involved in determining disease outcome in patients with *H. pylori* infection. HLA = Human leukocyte antigen; Hp = *H. pylori*; PUD = Peptic ulcer disease.

Two recent studies[558,559] have suggested that adhesin-receptor interactions may play a role in the topographical location of *H. pylori* in the stomach and hence disease outcome. Syder et al.[559,1055] showed that in normal mice the *H. pylori* isolate Hp1, which binds to NeuAcα2,3Galβ1,4 glycan receptors (recognised by the SabA *H. pylori* adhesin, Mahdavi et al.),[621] colonises primarily the part of the stomach that is devoid of parietal cells (a narrow band of pit cells positioned at the boundary between the squamous epithelium of the forestomach and the glandular epithelium). In transgenic mice where parietal cells are absent, Hp1 is also able to colonise the glandular

epithelium. This is because the sialylated glycan receptors are located in gastric units that are devoid of parietal cells (namely the junction between the squamous epithelium of the forestomach and glandular epithelium). When the parietal cells are absent from the epithelium, a new source of sialylated glycans becomes available to the bacterium and its niche for colonisation becomes enlarged. The parietal cells (and also their acid-production) thus act as 'gatekeepers' in *H. pylori* pathogenesis within the stomach.

The absence of parietal cells (and hence presence of NeuAcα2,3Galβ1,4 glycan receptors) in the antrum of the human stomach may partly explain why *H. pylori* preferentially binds to this region. However, once infection has established, in patients who develop severe chronic gastritis, parietal cells and acid production may be lost from the corpus/fundal regions of the stomach[1056]. In such cases *H. pylori* infection would be able to spread to these additional gastric regions because suitable receptors would have now become available for the bacteria to adhere to.

Akada et al.[558] found that when C57BL/6J mice were co-infected with two different strains of *H. pylori* (X47 and SS1), differences existed in the preferred gastric region that the strains colonised. X47 was found to be more abundant in the corpus whereas SS1 was more abundant in the antrum. They showed that this was partially due to the acid susceptibility (shown to be linked to cagPAI status)[1057] of the strains. SS1 (Cag+) grew better in mild acidic conditions (hence preferred colonising the antrum) and X47 grew better at pH 3.8 (more acidic, corpus-like conditions). They also suggested that the preference of the two strains to colonise different gastric regions may be due to their ability to adhere to particular glycan moieties on the gastric epithelium of the two niches and the results may reflect distributions of molecules that SS1 and X47 can use as receptors. Differences in distribution pattern were also shown to vary with changes in mouse genotype.

Very recently, one group of investigators has attempted to determine any differences in receptor distribution using the guinea-pig model of *H. pylori* infection.[1041] This group looked at the binding of lectins to sugars on the mucosal surface of different regions of the stomach, for example the sugars mannose, fucose, 2,3- and 2,6- linked sialic acid, N-acetylglucosamine and N-acetylgalactosamine/N-acetylglucosamine (GlcNAc and GalNAc/GlcNAc respectively) and complex type carbohydrates. They found that there was no difference in the receptors that were expressed in the canalis (antrum and pylorus) or body (fundus-corpus) regions of the stomach. There was also no difference between inflamed (*H. pylori* infected) and non-

inflamed (*H. pylori*-negative) stomachs. They concluded that guinea-pigs were different to humans because one study has shown that in humans different receptors are expressed in the fundus and antrum of the stomach and that a difference occurs between inflamed and non-inflamed tissue, especially with regarding to the expression of fucose.[1040] However, this is the only study that has looked at the human stomach.

The purpose of this study was therefore to investigate which bacterial adhesins and host cell receptors are involved in the adhesion of *H. pylori* to different parts of the human stomach, with the view to providing an indication of why *H. pylori* binds to either the antrum of the stomach (resulting in antral gastritis) or to the antrum, corpus and fundus (resulting in pangastritis) but is rarely seen in the fundus alone. If the presence of different receptors in the antrum and fundus are characterised, then suitable inhibitors of adhesion could subsequently be found, thus leading to the development of a potential treatment for clearing *H. pylori* infection from all areas of the stomach. The main *H. pylori* OMPs thought to be involved in adhesion of the bacterium to the human stomach are: BabA (which binds to Le b);[622] SabA (which binds to sLe x and sLe a)[621] and also AlpA, AlpB, OipA and HopZ (whose receptors are currently unknown).[676] These adhesins and receptors are therefore the main interactions under investigation in this study.

7.2 Methods

7.2.1 Microbial isolates

The following *H. pylori* strains were used in the study: J99 (WT) and three J99 mutants lacking the adhesins BabA, SabA and BabA/SabA;[53] kindly donated by Professor Thomas Borén, Umeå University, Umeå, Sweden); the guinea-pig-adapted *H. pylori* strain GP15 (WT) and four GP15 mutants lacking the adhesins: AlpA, AlpB, OipA and HopZ;[676] kindly donated by Professor Arnoud van Vliet, Erasmus MC-University Medical Center Rotterdam, Rotterdam, The Netherlands).

7.2.1.1 Characteristics of the strains

H. pylori strain J99 is a human isolate, which is cagA+/VacA(s1a/m1)+ and has been shown to bind to the receptors Le b, H1-containing glycoproteins, sLe x, sLe a, 3'SL, laminin, fibronectin, MUC5B (and others) in the human stomach. The BabA- mutant

has been shown to bind to the same receptors as J99 except for Le b and H1-containing glycoproteins; the SabA- mutant to Le b, H1-containing glycoproteins and MUC5B and the BabA-/SabA- mutant binds to none of the receptors except for laminin and fibronectin.[53,621]

GP15 is a guinea-pig adapted strain of *H. pylori* and is cagA+/VacA(s2/m2)+.[1058] The guinea-pig has been shown to be a good model of human infection, having several features common to the human stomach that are lacking in mouse model. For example, it has a cylindrical epithelium, is a sterile environment and produces IL-8.[1059,1060] Isogenic mutants of the guinea-pig-adapted *H. pylori* strain, GP15, were used in a study by de Jonge et al.[676] who found that WT, OipA- and HopZ- mutants still colonised the guinea-pig stomach but there was a significant reduction in colonisation by AlpA- or AlpB- mutants.

Growth conditions of *H. pylori* and labelling procedure are outlined in Chapter 2 (sections 2.1 and 2.2) and Chapter 3.

7.2.2 Tissue

A mixture of *H. pylori*-negative and *H. pylori*-positive biopsies of human stomach (formalin-fixed and embedded in paraffin wax) from 10 patients with gastritis (i.e. inflamed tissue), were obtained by Professor Dino Vaira. Biopsies were taken from both the antrum and fundus of each patient. Details of the patients and tissue are summarised in **Table 17**.

The tissue used for specificity experiments of the *H. pylori* mutants were biopsies from the antral region of the stomach of a Le a+/Le b- and a Le a-/Le b+ patient, both of whom were *H. pylori*-negative and had peptic ulcer disease. Stomach tissue was also taken from *H. pylori*-negative FVB/N (WT) mice and from *H. pylori*-negative FVB/N transgenic mice expressing the human Le b blood group antigen. In addition to Le b, the transgenic mouse has been shown to have *H. pylori*-sLe x binding.[621]

All human tissue used in this study was obtained and kindly donated by Professor Dino Vaira (University of Bologna, Italy) with the consent of the Ethics Committee, St Orsola Hospital, Bologna. The mouse tissue was kindly donated by Professor Lennart Hammerstrom, Karolinska Institute, Sweden). All tissues were formalin-fixed and embedded in paraffin wax.

PATIENT No.	AGE (yrs)	SEX	Inflammation		*H. pylori* positive		Chronic Gastritis	
			Antrum	Fundus	Antrum	Fundus	Antrum	Fundus
1	34	M	Y	Y	N	N	Y	
2	67	M	Y	Y	N	N	Y	
3	54	F	Y	Y	Y	N	Y	
4	58	F	Y	Y	N	N	Y	
5	53	F	Y	Y	Y	Y	Y	
6	47	M	Y	Y	Y	Y	Y	
7	73	F	Y	Y	N	Y	Y	
8	40	M	Y	Y	Y	Y	Y	
9	70	F	Y	Y	Y	Y	Y	
10	63	F	Y	Y	Y	Y	Y	

Table 17: Summary of patient details and the tissues used in this study. Black fields represent test not performed. M = male; F = female; Y = yes; N = no.

7.2.3 Determination of Le a and Le b phenotype in the antrum and fundus

Sections (5μm thick) were cut from the biopsies of the antrum and fundus of each patient. Immunohistochemistry was performed to look for the presence of Le a and Le b antigens. For details of method see Chapter 2 section 2.3.1 and Chapter 3 section 3.2.1.

7.2.4 Adhesion assay

After deparaffinising, antigen-retrieval steps were performed on the tissue sections. To expose the receptors required for BabA and SabA adhesion (i.e. for adhesion experiments using J99 and the BabA/SabA *H. pylori* mutants), sections were boiled for 5 mins in an 800W microwave in plastic coplin jars containing 15mls of Citrate buffer, pH 6.0.

Experiments were performed to optimise exposure of the stomach cell surface receptors required for *H. pylori* strain GP15 adhesion. After deparaffinising, sections from the stomachs of four patients (numbers 1, 2, 5 and 7) underwent two different treatments: digestion in α-chymotrypsin solution, pH 7.8 at 37°C for 10 mins or boiling for 5 mins in an 800W microwave in plastic coplin jars containing 15mls of citrate buffer, pH 6.0. No adhesion of GP15 was observed to the sections treated with α-

chymotrypsin, but a small amount of binding was seen to sections boiled in citrate buffer. A second experiment was thus performed, optimising boiling times with citrate buffer. Sections were boiled for either 5 or 10 mins in the microwave. Sections boiled for 10 mins were found to have much more binding than those boiled for 5 mins. Boiling sections for 10mins in citrate buffer was therefore chosen as the optimum antigen-retrieval method for exposing receptors required for adhesion of *H. pylori* strain GP15 (and mutants) to the mucosal epithelium of the stomach sections.

After antigen-retrieval, adhesion and adhesion-inhibition assays (using 250µg/ml Le b-HSA) were performed on the tissues. Details of the methods are described in Chapter 3 section 3.2.3 and 3.2.4.

7.2.5 Statistical analysis

Where appropriate, statistical analysis was performed on the data using either the unpaired t-test with the Welch correction (for parametric data with different standard deviations, SDs) or the Mann-Whitney U-test (for non-parametric data).

7.3 Results

7.3.1 Le a/Le b phenotype of patients' tissue

7.3.1.1 Lewis antigens

Eight of the ten patients (numbers 1-4 and 7-10) were found to be Le b+/Le a- and the remaining two (patient numbers 5 and 6) were Le b+/Le a+. For all ten patients the same Lewis phenotype found in the antrum was also expressed in the fundus.

7.3.2 Specificity of *H. pylori* BabA/SabA mutants

To test the specificity of the BabA/SabA *H. pylori* mutants, an adhesion assay was performed on human stomach sections from patients expressing either the Le a or Le b phenotypes as well as on stomach sections from the WT and Le b transgenic mouse. The results of these experiments are shown in **Figure 51**.

For the WT strain of *H. pylori* (J99), adhesion was highest to the human Le b stomach followed by the Le b transgenic mouse, as expected. Adhesion to the Le a human stomach was lower and only a residual binding was observed on the WT mouse stomach. The BabA- *H. pylori* strain showed almost no adhesion to all the tissues except for the Le b human stomach, which was lower compared to the adhesion of the WT strain to the same tissue. The *H. pylori* mutant lacking the SabA adhesin showed the same pattern of binding as the WT strain, except adhesion to the Le b human stomach was lower, although not significantly ($p>0.05$). Binding of the mutant lacking both the BabA and SabA adhesins followed the binding of the BabA mutant.

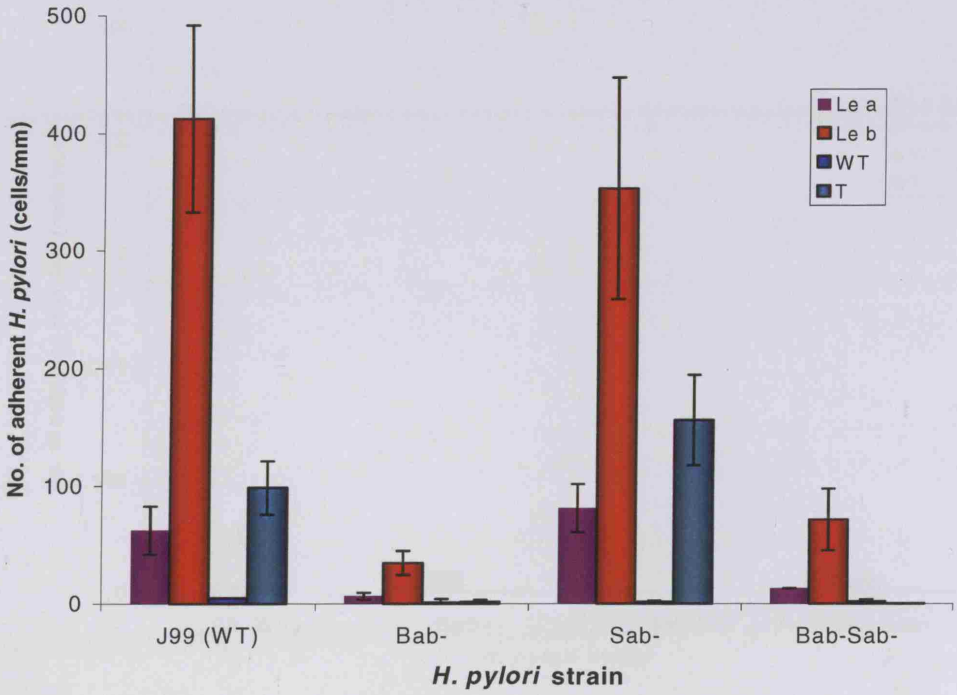

Figure 51: Adhesion of BabA/SabA mutants of the J99 strain of *H. pylori* to stomach sections. Le a = stomach sections from humans expressing Le a phenotype; Le b = stomach sections from humans expressing Le b phenotype; WT = stomach sections from WT mouse; T = stomach sections from transgenic mouse expressing the human Le b phenotype. Experiments were performed three times; the mean of the three experiments +/- S.E. is shown.

The results of the specificity experiments described above were confirmed in experiments using Le b-HSA as an inhibitor of adhesion. As expected, by pre-incubating the *H. pylori* mutants with Le b-HSA, the binding of the WT and the SabA- strains was inhibited to all the tissues they bound to **(Figure 52).** These experiments also confirmed that the WT (J99) strain binds to tissue mainly via Le b antigens and that adhesins other than SabA are involved in adhesion, since some binding of the WT strain, BabA- mutant and BabA-/SabA- mutant was observed on the Le b human stomach when incubated with Le b-HSA.

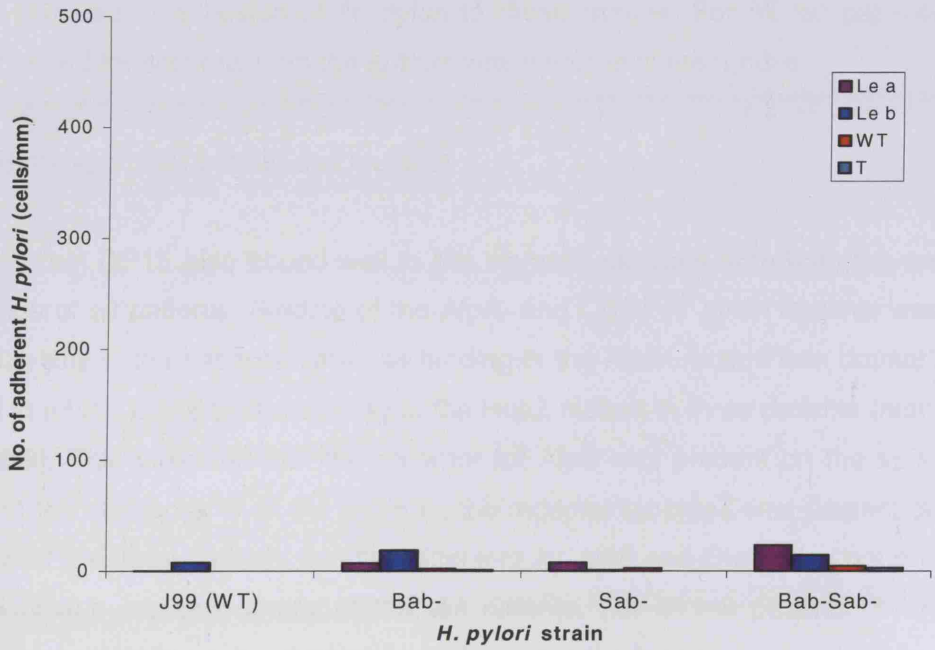

Figure 52: Inhibition of adhesion of BabA/SabA mutants of the J99 strain of *H. pylori* to stomach sections using 250µg/ml Le b-HSA. Le a = stomach sections from humans expressing Le a phenotype; Le b = stomach sections from humans expressing Le b phenotype; WT = stomach sections from WT mouse; T = stomach sections from transgenic mouse expressing the human Le b phenotype. Experiments were performed twice; the mean of the two experiments is shown.

7.3.3 Adhesion of *H. pylori* mutants to the antrum and fundus of inflamed human stomachs

7.3.3.1 Binding of J99 strain and mutants

The WT strain J99 bound well to sections from the antrum and fundus of all patients. Binding of the BabA- *H. pylori* mutant was greatly reduced in all ten patients (compared to the WT strain), indicating that they expressed the Le b antigen; confirming the IHC results. Binding of the SabA- mutant was not reduced in eight of the patients (numbers 1-4 and 7-10), indicating that the receptor for SabA was not present or SabA was not a major adhesin involved in adhesion to the tissue, or its binding is masked by the strong binding of BabA to the Le b antigen. Whereas in the remaining two patients (numbers 5 and 6) binding of the SabA mutant was reduced, indicating that the SabA receptor was present on the epithelial surface or was an adhesin involved in adhesion of *H. pylori* to these tissues. For all ten patients the result obtained for sections from the antrum was mirrored in the fundus.

7.3.3.2 Binding of GP15 strain and mutants

The WT strain GP15 also bound well to the stomach sections from both the antrum and fundus of all patients. Binding of the AlpA- and OipA- *H. pylori* mutants was not reduced in any of the patients, whereas binding of the AlpB- mutant was dramatically reduced in all ten patients and binding of the HopZ mutant in three patients (numbers 6, 7 and 9). This indicated that the receptor for AlpB was present on the epithelial surface of the stomachs of all the patients, the receptor for HopZ was present on the stomachs of the three patients and the receptors for AlpA and OipA were not present on the stomach mucosa of any of the ten patents. For all ten patients the result obtained for sections from the antrum was mirrored in the fundus.

Summary

The results of the immunohistochemistry and adhesion experiments on the antral and fundal biopsies from the ten patients, are summarised in **Table 18.**

PATIENT No.	RECEPTORS PRESENT													
	Antrum							Fundus						
	Le a	Le b	sLe x	AlpA	AlpB	OipA	HopZ	Le a	Le b	sLe x	AlpA	AlpB	OipA	HopZ
1		*			*				*			*		
2		*			*				*			*		
3		*			*				*			*		
4		*			*				*			*		
5	*	*	*		*			*	*	*		*		
6	*	*	*		*		*	*	*	*		*		*
7		*			*		*		*			*		*
8		*			*				*			*		
9		*			*		*		*			*		*
10		*			*				*			*		

Table 18: Details of the presence of cell surface receptors in stomach biopsies from the fundus and antrum of ten *H. pylori*-negative patients. Because the specific receptors for the *H. pylori* adhesins SabA, AlpA, AlpB, OipA and HopZ were not determined, the names of the adhesins are written in their place.

7.4 Discussion

H. pylori infection is most often confined to the antrum of the stomach where it results in antral gastritis and may lead eventually to the development of duodenal ulcer. In some patients, *H. pylori* colonisation spreads throughout the stomach to include the body and the fundus, resulting in a pangastritis, which, in a few cases, can lead to the development of gastric malignancies.[551] It is very rare however, for *H. pylori* to colonise the fundus alone. Although there are several factors that are thought to affect the pattern of *H. pylori* distribution, the adhesin-receptor interactions involved in the colonisation of *H. pylori* to different regions of the stomach has been largely unexplored. This study has therefore looked at the presence of *H. pylori* receptors on the mucosal surface of the antral and fundal regions of the inflamed stomachs of ten patients, in order to try and offer an alternative explanation for the topographical differences seen in *H. pylori* colonisation of the human stomach. Several *H. pylori* adhesins are thought to be involved in adhesion of *H. pylori* to the human stomach and these include BabA, SabA, AlpA, AlpB, OipA and HopZ. [621,622,676,679,681,962] Mutants lacking these adhesins have therefore been used in binding assays to determine the presence of their complementary receptors in the patients' stomachs.

The binding specificity experiments for the *H. pylori* mutants of strain J99 (lacking the BabA and/or SabA adhesins) to the gastric tissue of humans, expressing either the Le a or Le b antigens and WT or Le b transgenic mouse, showed that using these mutants was a good indicator of the presence of the Le b antigen. Binding of the BabA- mutant was highly significantly reduced ($p<0.01$) from the WT strain J99 in the tissues expressing Le b (Le b transgenic mouse and Le b human) showing that BabA was involved in J99 binding and the Le b receptor was present in these two tissues as expected. Moreover, the Le b antigen was the principal receptor used by J99 to bind to stomach tissue. Adhesion of the BabA mutant to the Le a tissue was also reduced. It may be therefore that the Le a tissue expressed a small number of Le b receptors or the loss of the BabA protein from the bacteria affected the binding of other adhesins. Perhaps some adhesins are co-localised or adjacent to each other and binding occurs via their interactions with each other and thus loss of BabA interfered with their binding ability.

The results also show that adhesins other than BabA are involved in the binding of J99 since the BabA and BabA/SabA mutants were still able to bind to human tissues expressing Le b antigens. From these results, it is difficult to conclude if SabA plays a role in adhesion since the SabA mutant bound strongly to tissues expressing Le b antigens. It is possible that SabA does play a role but its binding is masked by the binding of *H. pylori* to the Le b antigen. The results do however, confirm the findings of Walz et al.[53] who have shown that strain J99 and its SabA- mutant both bind to Le b-containing neoglycoproteins and J99 binds to other receptors such as sLe a, sLe x, laminin and fetuin fibronectin; the BabA- mutant binds to sLe x and sLe a and laminin and fetuin and of all these receptors the BabA-/SabA- mutant binds only to lactoferrin and fibronectin. In a previous study, Mahdavi et al.,[621] have shown that *H. pylori* strain J99 and its BabA2- mutant do bind to inflamed tissue and therefore because the human tissues in this study were inflamed, the BabA-mutant would be expected to also bind. However in this study, binding of the BabA- mutant to Le b mouse tissue was lost and binding to the human tissue was greatly reduced. This suggests the binding that does occur is due to the involvement of other *H. pylori* adhesins, such as SabA.

These results therefore suggest that BabA-Le b binding is the prominent adhesin-receptor interaction occurring in all the tissues, and perhaps that binding to Le b is stronger than that of SabA to its receptors sLe x or sLe a. Odenbreit et al.[39] have suggested that the strong Le b attachment of *H. pylori* to gastric tissue, might cover other attachment properties. There may also be far less receptor molecules for SabA (than there are receptors for Le b) on the epithelial surface of the Le b human and

mouse stomachs. Because some binding to the human stomach tissue still occurred with the BabA-/SabA- mutant, this indicates that binding to the human tissue involved the use of adhesins other than BabA and SabA. Binding of an *H. pylori* BabA-/SabA- mutant on monkeys has been shown to correlate with sLe x but not sLe a.[982] However, 50% of strains that bind sLe x also bind sLe a.[621]

The overall results of the adhesion-specificity experiments suggest that adhesion of *H. pylori* strain J99 to stomach sections is mainly mediated by its BabA adhesin interacting with Le b and there may be some involvement of SabA and other adhesins.

In order to determine any differences between *H. pylori* receptors present in the antrum and fundus of the human stomach, biopsies from the antrum and fundus of ten *H. pylori*-positive and -negative patients with gastritis (i.e. inflamed tissue) were tested for the presence of Le a and Le b antigens by immunohistochemistry and the receptors for *H. pylori* BabA, SabA, AlpA/B, OipA and HopZ were investigated by observing the binding of mutants lacking these adhesins, to the patients' tissues.

The results of the immunohistochemistry and adhesion experiments showed that in the present study, all ten patients were found to express the Le b receptor and the other two of these patients (numbers 5 and 6) also expressed Le a. Only these two patients (who expressed the Le a receptor) also expressed the receptor for SabA (i.e. binding of the SabA- mutant was reduced). It is interesting that the SabA- mutant bound to all the Le b+/Le a- patients. One may have expected the SabA- mutant to have shown reduced binding since all the tissues were inflamed and the SabA receptors (sLe x and sialylated glycoconjugates) have been shown to be associated with inflamed tissue.[621,982,983] Expression of sialylated derivatives of the Lewis antigens have also been shown to be considerably reduced in patients with normal gastric tissue.[532] This indicates that the SabA receptor was either not present on the stomach tissue of these patients or was not a major adhesin involved in the adhesion of *H. pylori* to the stomach. However, as suggested by the results of the specificity experiments, the binding of *H. pylori* to the SabA receptor may have been masked by the BabA-mediated binding of the organism to Le b.

de Bolos et al.[1061] showed that the type of Lewis receptors present in the gastric mucosa depends upon which mucin type is present in the patient. They found that MUC5AC+ cells express both Le b and sLe a. Sakamoto et al.[532] also showed that sLe a was not always specific to Le a stomachs; it was also expressed in normal Le

a+/Le b+ stomachs. The co-expression of Le b, Le a, sLe a and sLe x has been shown in the antrum and fundus of *H. pylori*-negative rhesus monkeys and all Le b+ animals were also Le a+.[982] Usually individuals are either Le b+/Le a- or Le b-/Le a +. However a Le b+/Le a+ phenotype can occur in individuals when the expression of the H1 Lewis structure is reduced due to a mutation in the FUT2 (fucosyl-transferase 2) enzyme, resulting in weak enzymatic activity.[1062,1063]

The presence of the SabA receptor (sLe a or sLe x) in the two patients in this study, may be indicative of the presence of GC or premalignant phenotypes such as intestinal metaplasia (IM) since both sLe a and sLe x have been shown to be markers for GC and aggressive tumours.[1064-1068] It may also be therefore that the Le a+/Le b+ phenotype seen in the two patients in the present study was the result of the presence of GC. The presence of Le a in Le b+ patients (i.e. Le a+/Le b+ phenotype) may also be indicative of GC since Sakamoto et al.[532] found that in secretor individuals (i.e. Le b+), Le a was only expressed in patients with GC and sLe a (which was present in some normal stomachs) was more pronounced in patients with GC. Although the presence of IM or GC was not noted as a histological observation in the patient details, the detection of specific antigens (such as sLe a or sLe x) as performed in the present study, may be a more accurate determinant of the early stages of IM or GC development.[1069,1070]

The two possible receptors for SabA are sLe x and sLe a.[621] It seems in this study (although the sample size is too small to be conclusive), that the SabA receptor is associated with the presence of a Le a+/Le b+ phenotype. This is in line with previous findings which have shown that sLe a is generally restricted to the MUC5AC-producing cells, (MUC5AC also expresses Le b), of Le a individuals.[532,1061,1064,1071] *H. pylori* infection has been shown to induce sLe x and increase sLe a expression in the gastric epithelium of humans and the rhesus monkey,[57,621,951] which may enable *H. pylori* to spread after it has established and adhered to initial receptors present such as Le b.

In order to determine for certain which receptor is present in the stomachs of these patients, immunohistochemistry needs to be performed looking firstly for whether sLe x or sLe a are present, and secondly if sLe a is detected, which derivative is present, since of the two sialylated derivatives of Le a, it has been shown that the 2-3sLe a derivative in particular (rather than 2-6sLe a derivative), is the more important/specific marker of GC.[1072,1073] Additionally, in order to determine which receptor is involved in SabA-mediated adhesion, the WT and SabA- *H. pylori* strains

could be preincubated with sLe x or sLe a and observed to see whether adhesion is reduced. If sLe a is the receptor for the *H. pylori* SabA adhesin in the patients in this study, it may explain why the receptor for SabA is only present in patients who are Le a+ and Le b+

The present study has also shown that not all patients express the same profile of receptors for *H. pylori*. It has been shown that there is considerable variation in blood-group and sialic acid expression in humans and monkeys.[1074,1075] Odenbereit et al.,[39] using gastric tissue sections from the antrum of human biopsies, found that expression of receptors on the epithelial cell surface of different biopsies is variable, since adhesion of a distinct *H. pylori* strain was variable on sections from different donors. Mollicone et al.[982] and Linden et al.[1075] found that even within the same human gastric tissue, areas exist which are Le b+ or Le b-.

It is known that Le b is generally expressed on the gastric epithelial surface,[532] but a substantial subset of *H. pylori* strains do not produce the BabA protein (28-62% of isolates investigated),[604,1076] suggesting that other major adhesin-receptor interactions are involved in *H. pylori* adhesion to the stomach. This study therefore investigated the presence of additional receptors to Le b.

The results of the immunohistochemistry and adhesion experiments showed that all ten patients expressed the receptor for AlpB and three of the ten also expressed HopZ (one of which was Le a+), while no patients expressed the receptors for AlpA or OipA. The most common phenotype was Le b+/AlpB receptor+ and this was found in six of the ten patients. The results of this study confirm previous findings that AlpB and HopZ (but not OipA) are involved in adhesion of *H. pylori* to human and animal gastric tissue.[39,676,681,1077] Odenbreit et al.[677] showed that AlpA- and AlpB- mutants did not bind to gastric biopsies. They observed that AlpB at least was required for adhesion and AlpA may not be needed. Peck et al.[681] using an adhesion assay with human AGS (adeno gastric carcinoma) cells, showed a significant decrease in binding by a HopZ- mutant compared to a WT *H. pylori* strain. De Jonge et al.,[676] using the same *H. pylori* strain (GP15) and its mutants lacking the *alpA, alpB, oipA* and *hopZ* genes (as used in this study), found the same pattern of results in the guinea-pig as the present study did in humans: that OipA and Hop Z were not involved in the adhesion of *H. pylori* to the antral region of the stomach, but AlpA and B were important in colonisation. They concluded that Alp proteins play an active role in establishing and maintaining gastric colonisation because the *alpA* gene is known to

be transcribed in *H. pylori* strains *in vivo* in human gastric tissues and murine gastric tissues in the first three months of infection.[1078]

However, Yamaoka et al.[682] using C57BL/6 mice in a mouse model of *H. pylori* infection showed that if two or more of the *OipA, HopZ, HopO* and *HopP* genes were switched off, colonisation rate was markedly reduced, indicating a role for OipA in *H. pylori* adhesion. There was a close relationship between *H. pylori* density on the gastric mucosa of humans and mice when using same *H. pylori* strains. They also showed that reduced expression of OipA, SabA, SabB and HopZ limited the colonisation of the murine stomach when two or more of these OMPs were not expressed. Interestingly, AlpA has also been shown to be involved in *H. pylori* adhesion to the human stomach. Odenbreit et al.[39] looked at the effect of an AlpA- strain on adhesion and found that removing AlpA- also abolished adhesion and thus expression of neither *alpA* nor *alpB* alone might be sufficient for binding of *H. pylori* to the epithelial cell surface. Their data suggest that AlpA and AlpB are necessary for the adherence phenotype but they found that AlpB alone does not enable adherence since AlpB reconstituition (after it was initially removed from *H. pylori* cells), could not restore binding of the bacterium. But this was not the case in the present study. This may be due to a difference in the patients used in the two studies, inflamed tissue was used in this study whereas in the studies of Odenbreit et al.[39] the tissue may have been non-inflamed; this important detail is not mentioned in the paper. It may be that the receptors for AlpB and OipA were not actually present on the gastric epithelial surface of the ten patients in the study. Perhaps AlpA is only found in non-inflamed tissue or perhaps the antigen-retrieval technique employed in this study was not sufficient to unmask the AlpA receptor from the formalin-fixed tissue.

Overall, the results of the immunohistochemistry and adhesion experiments of the *H. pylori* mutants showed that each patient expressed the same receptors in both the antrum and fundus of their stomach. This is a similar result to that found in the study of Lueth et al.[1041] who found that in the guinea-pig model of *H. pylori* infection, there was no difference between the receptors present in different topographical regions of infected and non-infected (inflamed) stomachs. This may be unique to the guinea-pig model as new receptors such as sialylated glycans have been shown to be upregulated in human and rhesus monkey infection and in inflamed tissue.[620,621,983] However, a difference has been shown to occur in the fundus and antrum of the human stomach[1040] and the animal studies of Akada et al.[558] and Syder et al.[559] also suggest a difference. The reason that no difference was found in the present study may be because all the tissues used were inflamed. The study of Akada et al.[558] and

Syder et al.[559] used mice with non-inflamed (*H. pylori*-negative) stomachs. Baczako et al.,[1040] using human stomachs also showed that there was a difference in receptors (but not sialic acids) expressed in the body and antrum of non-inflamed stomachs. However, of importance is their finding that like the present study, there was no difference in the receptors expressed in different regions (antrum and body) of inflamed stomachs. The receptors they looked for on the stomach were: L-fucose; D-mannose; D-glucose; galactosyl *N*-acetylgalactosamine; D-*N*-acetylgalactosamine; D-galactose and silaic acids.

Of significance therefore is the statement of Lueth et al.[1041] that the region-specific differences in receptors in non-inflamed tissues, may explain the different susceptibilities of the different regions of the stomach to *H. pylori* infection. If suitable receptors are not present in the fundus of a non-inflamed stomach, this may therefore offer an alternative explanation to acid secretion, for why *H. pylori* initially and preferentially colonises the antrum of the stomach. Once *H. pylori* has colonised the antrum, however, the resulting inflammation, favourable reduction in acid in the gastric body and expression of new receptors, may allow *H. pylori* infection to spread and to colonise additional regions of the stomach, such as the body and fundus. Of course there are multiple factors which affect *H. pylori* colonisation of the stomach such as host genotype, genotype of the infecting strain, strength of the individual host's immune response and so on.

In conclusion, this study is of importance because it is a preliminary investigation into the presence of *H. pylori* receptors in different regions of the human stomach, which has been rarely studied before. It has brought to light several interesting questions which remain to be answered that may also be important regarding the pathogenic process and treatment of *H. pylori* infection. The results seem to suggest that the same receptors necessary for *H. pylori* adhesion are present in both the antral and the fundal regions of inflamed human stomachs. This may indeed be the case but further studies involving larger numbers of patients would be required to confirm this. It may be interesting to compare the expression of receptors in the antrum and fundus of inflamed vs non-inflamed tissues and see whether the presence of inflammation results in a difference.

The results of the present study have also suggested that the SabA receptor (sLe a or sLe x) was expressed only in patients that were Le a+/Le b+. Further studies involving immunohistochemistry on larger numbers of patients are required to

determine the exact receptor for the SabA adhesin in these patients and to determine therefore whether sLe x or sLe a is associated with Le a expression, or whether in these patients sLe a or sLe x is simply a marker indicative of GC.

Since it has been shown that inhibitors of adhesion can be used to prevent the binding of *H. pylori* to the stomach (**Table 11** and Chapter 5) and because there are multiple *H. pylori* receptors present in the human stomach, once the phenotype of the patient's stomach has been determined, treatment for *H. pylori* infection in the future could involve the use of several different inhibitors targeting different adhesin-receptor interactions. If the same receptors are present in both the antrum and fundus of patients with inflamed stomachs, as this study has shown, then a single treatment consisting of a smaller, specific combination of receptor analogues, could be effective at treating *H. pylori* infection in both the antral and fundal regions of the stomach.

SECTION IV

DISCUSSION

Chapter 8

DISCUSSION

Due to the ever-increasing development of antibiotic resistance in microbial pathogens such as *H. pylori* and *C. albicans*, especially over the last 10 years, there is a greater need than ever before for the development of alternative therapies to treat infection caused by these microorganisms. Inhibition of microbial adhesion is one such method, which circumvents the problems of resistance associated with treatments involving antibiotics and other such agents which have a static or cidal effect on microorganisms. The main objectives of the present study were to develop a new and accurate method for assessing the inhibition of microbial adhesion and use this method to investigate the effectiveness of potential and novel inhibitors of *H. pylori* and *C. albicans* adhesion; namely carbohydrates, domain antibodies, minibodies and plant extracts. Additionally, because *H. pylori* selectively colonises specific topographical regions of the human stomach, the final objective of this study was to determine whether different adhesin-receptor interactions were responsible for this phenomenon, with the view to developing a suitable anti-adhesive therapy which would be able to clear *H. pylori* infection from all regions of the stomach.

Development of quantification method

An adherence assay using tissue sections from human stomach biopsies and pieces of rat vagina was optimised for *H. pylori* and *C. albicans* adhesion respectively. This represented a more life-like model than those used in most studies in the literature, (which usually involve the use of cell lines or monolayers), in order to investigate potential inhibitors of *C. albicans* and *H. pylori* adhesion. Because the assay in this study used fluorescently-labelled organisms and tissue, sections could be observed using a confocal microscope and digital images taken.

Previous studies, investigating microbial adhesion or inhibitors of microbial adhesion, commonly used manual methods such as microscopic counts, for quantification. In order to develop a faster and more accurate quantification method to assess adhesion and inhibition of adhesion, in this study, several image analysis software packages were compared in order to find the most suitable one and different methods of quantification were developed for each package. Of all the software

packages and methods utilised, the best and most accurate for quantification was found to be 'Metamorph' software using the 'Region of Interest' (ROI) method with standard area method of counting. Although some of the software packages tested in this study were used in the past for the same purpose, the accuracy, speed and ease of use of these packages was found to be inferior to Metamorph and the ROI method developed in this study. One major reason for this was the unique ability of Metamorph to be able to accurately count the number of individual cells contained in a cluster. Having found and developed the optimal assay and method of quantification, this system was applied to demonstrate and quantify the effectiveness of potential inhibitors of *C. albicans* and *H. pylori* adhesion.

Novel inhibitors of *C. albicans* adhesion

A number of domain antibodies (dAbs) against the Sap2, MP65 and enolase proteins of *C. albicans* were tested in this system for their potential as anti-adhesins. Of these dAbs, anti-Sap2 dAbs 4A7, 4A14 as well as anti-MP65 dAbs 3-1, 3-2 and 3-6 and anti-enolase dAbs 5, 6, 9 and 11 were shown to be most effective, inhibiting adhesion of *C. albicans* to rat vaginal tissue sections between 58% - 99%. Interestingly, the same dAbs (4A7, 4A14, 3-1, 3-2 and 3-6) were also shown to be effective in an *in vivo* rat model of vaginal candidasis.[950] This demonstrates the usefulness of the *in vitro* model developed and used in the present study, as a fairly accurate indicator of potential inhibitors of *C. albicans* adhesion. The use of dAbs as inhibitors of microbial adhesion is a novel application for these antibody fragments and the results of this study show that the proteins which the dAbs target are involved and important in the adhesion of *C. albicans* to the rat vagina. Using dimers comprising two of the successful dAbs (3-6 and 4A7) was shown to be more effective than using the monomers alone, which is probably attributable to the dual antigen-targeting capability of the dimers. It also demonstrates that a single adhesin alone is not responsible for adhesion but rather interactions involving multiple adhesins are likely to occur. The dAbs used in this study could form the basis of an effective and novel treatment for vaginal candidiasis.

Novel inhibitors of *H. pylori* adhesion

By using carbohydrates, dAbs, minibodies and bovine colostrum (BC), the adhesion of *H. pylori* to gastric tissue sections was inhibited. The most successful inhibitors were

glycoconjugates and dAbs that were analogues of the Le b receptor (targeting the BabA adhesin), namely Le b-HSA, Le b(hexa)PL and dAb 25. The best inhibitor was found to be Leb(hexa)PL, the success of which is probably attributable to its multivalent structure, allowing it to bind to multiple BabA adhesins at one-time. BC and sLe x also inhibited adhesion and because these were known and thought to target different adhesins of *H. pylori*, they were tested in combination with the successful anti-BabA inhibitors, with the view to producing a more effective inhibitor because it would target and block more than one adhesin at the same time. This hypothesis was shown to be correct: the combinations of inhibitors were found to be more successful than when the inhibitors were tested on their own; higher percentages of inhibition were achieved using lower concentrations. The best combinations were Leb-HSA + sLe x and Le b(hex)PL + sLe x which gave almost 100% inhibition using a lower concentration than the individual glycoconjugates alone. Again, the success of this combination was probably due to the multivalent nature of the Le b(hexa)PL.

The results confirm the findings of previous studies which have shown that sLe x, Le b-HSA and bovine colostrum can inhibit *H. pylori* adhesion.[15,621,668] However the use of Le b(hex)PL, minibodies and dAbs as well as combinations of the inhibitors are novel applications.

In addition to inhibiting adhesion, the glycoconjugates Le b-HSA and Le b(hex)PL were also shown to be able to remove *H. pylori* from gastric tissue sections once it had already bound. This suggests that *H. pylori* has a higher affinity for the inhibitors than for the receptors present on the gastric mucosa, probably because the inhibitors are multivalent analogues of the gastric receptor and are thus chemically more attractive to the *H. pylori* adhesins. Very few studies in the literature have investigated the ability of inhibitors to remove bound *H. pylori*. [665,964,973] The present study therefore, is of importance because it demonstrates not only the prophylactic treatment potential of the inhibitors but also a therapeutic application, which is more applicable to the clinical scenario. Taken together, the findings of this study demonstrate a plausible and potential novel treatment for *H. pylori* infection involving the use of combinations of anti-adhesins, which are able to both inhibit and remove *H. pylori* from the gastric mucosa. The finding in this study that using combinations of inhibitors improves efficacy and requires lower concentrations, also indicates the economic benefits of this potential treatment. Of course, these findings will have to be confirmed *in vivo*.

Plant extracts - novel sources of anti-*H. pylori* agents

Plants and plant extracts have been used for thousands of years as natural treatments for numerous physical ailments and diseases. Many studies in the literature have shown that plants are an alternative source of antimicrobial agents that are effective against many different types of bacteria and fungi.[831,832] A number of studies have shown that certain plants contain substances that have antibacterial effects on *H. pylori,* however, their ability to inhibit adhesion has infrequently been studied. In the present study, the extracts of 25 plants (made by boiling the plants in water), which are commonly used for medicinal or culinary purposes, were tested for bactericidal and anti-adhesive activity against seven different strains (type and clinical) of *H. pylori.* 17 plants were found to have bactericidal activity, killing all organisms within 60 minutes of incubation. Turmeric was the most efficient and killed all seven strains within 15 minutes; ginger, cumin and chilli were the next best, killing all strains within 30 minutes.

Turmeric, borage and parsley, which all had cidal effects on *H. pylori*, were also found to inhibit the adhesion of *H. pylori* to gastric tissue sections. These two effects were shown to be independent of each other, since the cidal effects of the plants were not attributable to lysis. A combination of the successful plant extracts (turmeric, borage and parsley) was shown to be more effective at inhibiting *H. pylori* adhesion than when the extracts were used separately. This was probably because the extracts were found to target different *H. pylori* adhesins: parsley seemed to inhibit mainly BabA whereas turmeric and borage seemed to inhibit adhesins other than BabA. The combination of the extracts therefore simultaneously targeted multiple adhesins, thus having a synergistic effect on inhibition.

Turmeric, ginger and chilli have previously been shown to have bactericidal effects on *H. pylori.*[1018-1020] However, the cidal effects of cumin and the cidal and anti-adhesive effects of borage, turmeric and parsley are new findings. This study is of importance because it indicates a possible new therapy for *H. pylori* infection, which is an alternative to antibiotic treatment and avoids the problems associated with the development of resistant organisms. Using extracts of plants obtained by boiling is also of importance because it makes this potential therapy cheap to manufacture as well as accessible to persons in the developing world, for whom *H. pylori* infection (and resistance to antibiotics) is more of a problem.

H. pylori adhesion - variations with gastric region

It is not fully known why *H. pylori* initially and preferentially colonises the gastric antrum and in some people is able to spread to the body and fundus, but rarely colonises the fundus alone. Several hypotheses have been proposed and it has been shown that acid-regulation has a major role to play in the topographical distribution of the organism. However, no single factor alone is sufficient to explain the distribution patterns seen. Very little work has been done investigating the adhesin-receptor interactions involved in adhesion of *H. pylori* to different regions of the stomach and whether differences in receptors within the gastric compartments may be involved in determining the distribution of *H. pylori* infection.

By performing immunohistochemistry and observing the binding of *H. pylori* mutants to tissue sections, the presence of *H. pylori* receptors in the antrum and fundus of patients with inflamed stomachs was investigated. The study was of importance because the presence of receptors in stomach regions has rarely been studied before. The results suggested that the same receptors necessary for *H. pylori* adhesion are present in both the antral and the fundal regions of inflamed human stomachs, as has been shown in a study by Baczako et al.[1040] However, further studies involving larger numbers of patients are required to confirm this and studies using non-inflamed tissue must be performed to determine whether differences in receptor expression exist between inflamed versus non-inflamed tissue.

All ten patients were found to express the Le b and the AlpB receptor and the other two patients also expressed Le a. Only these two patients (who expressed the Le a receptor) also expressed the receptor for SabA. Three of the ten also expressed the HopZ receptor (one of which was Le a+), while no patients expressed the receptors for AlpA or OipA. The most common phenotype was Le b+/AlpB receptor+ and this was found in six of the ten patients. These results confirm previous findings that AlpB and HopZ (but not OipA) are involved in adhesion of *H. pylori* to human and animal gastric tissue.[39,676,681,1077] The results of the present study also suggest that the SabA receptor (sLe x or sLe a) was expressed only in patients that were Le a+/Le b+. Further experiments are required to determine the exact receptor for the SabA adhesin in these patients and whether sLe x or sLe a is associated with Le a expression, or whether in these patients sLe x or sLe a is simply a marker indicative of gastric cancer.

Conclusion

In this study, a number of effective agents have been identified with the ability to inhibit *H. pylori* and *C. albicans* adhesion. The fact that some of these inhibitors, which were shown to be effective in the *in vitro* system developed in the present study, were also shown to be effective *in vivo,* adds weight to the results of this study and indicates that treatment involving the use of these agents may be a plausible possibility in the future.

This potential therapy would be an alternative to antibiotic treatment and has a major advantage over antibiotic therapy: by sterically hindering adhesion rather than killing the organism, it circumvents the associated development of resistant organisms. One limitation of using anti-adhesins however, as for antibiotics, is the ability of the agent to access mucosal microbial pathogens since they are usually located beneath the mucus layer. However, mucolytic agents or agents which down-regulate the synthesis and reduce the viscosity of mucin, could be used in combination with anti-adhesins, a practice which is commonly employed during antibiotic therapy.[1079,1080] Interestingly, immunisation experiments with microbial adhesins have demonstrated the potential for the generation of antibodies that inhibit adhesion, for example in *E. coli* and *C. albicans*.[950,1081-1083] It now remains for the effectiveness of anti-adhesins such as those identified in the present study, to be demonstrated in human trials.

Limitations of the study and future work

Limitations of the study included:

1. Using gastric tissue that had been formalin fixed, despite antigen-retrieval methods, was a limitation of the *H. pylori* studies. Frozen human stomach tissue was not available. For the *C. albicans* studies frozen vaginal tissue was able to be used, however, the tissue was from rats because human vaginal tissue could not be obtained, which is also a limitation.

2. pH. The assays were performed at a neutral pH to test the inhibitors. It may have been better to test the agents a pHs the same as those found in the gastric and vaginal environments, however the inhibitors were going to be tested in animals after the *in vitro* stage. This would therefore serve as a better

indicator of how the inhibitors survive the harsh environmental conditions and the process of digestion, rather than simply a change in pH. Additionally the pH at the mucosal- epithelial surface has been shown to be nearer to neutral, since the mucus barrier forms a protective layer from acid produced in the stomach and vagina.

3. Temperature. All assays were carried out at room temperature, which is not representative of the human body which has a core temperature of 37°C. However, it is the temperature and pH at which the organisms are grown at (not of the assay) which affect their binding, since these affect the expression of cell surface adhesins, morphology (i.e. hyphal growth in *C. albicans*) and cell surface hydrophobicity.[259,323,1084,1085] All organisms used in the study were grown at 37 °C and were dead when actually used in the assays.

4. Number of experimental repetitions. For all inhibitors it would be better to have tested on tissues from several different patients/rats (larger sample sizes) and to repeat the experiments several times (in the case of *C. albicans* inhibitors). This would reduce error margins, increase statistical power and introduce sample variation, which would give a more representative view of how the inhibitors might work in individuals, all of whom would show genotypic and phenotypic variation in their tissues. Unfortunately, time and the amount of inhibitors available for testing were limited, not allowing this to be performed.

5. Controls. For some experiments, relevant negative controls were not available; the use of these would have enabled a more accurate analysis of the efficacy of the agents in question.

6. Regions study. This study was limited because only inflamed human gastric tissue was used (non-inflamed human gastric tissue being unavailable) and therefore regional differences in the expression of *H. pylori* receptors in the normal human stomach, which would show why *H. pylori* may preferentially colonise the antrum, could not be studied. Additionally, the study was limited by only using mutants of *H. pylori* lacking adhesins, in order to demonstrate the presence of AlpA/B OipA and HopZ receptors, since the exact specificity of the mutants for the receptor epitope was uncertain.

Future experiments:

1. Repeat experiments alongside appropriate additional controls.

2. Test all the *H. pylori* inhibitors and plants *in vivo*.

3. Fractionate the plant extracts to find the component responsible for inhibition of adhesion. These could then be purified and tested to produce a more potent therapeutic agent.

4. For the regions study, the same experiments could be repeated using antibodies against the adhesins for AlpA/B OipA and HopZ, to confirm the presence of receptors for these adhesins by immunohistochemistry. Experiments to determine the exact receptor for SabA (sLe a or sLe x) would also be important. Also, it would be important to test inflamed and non-inflamed human stomach tissue to see whether differences in receptor expression in the stomach regions occur due to inflammation.

5. Use of different anti-adhesins. Treatment of *H. pylori* infection in the future could involve the use of several different inhibitors targeting different adhesion-receptor interactions. If the same receptors are present in both the antrum and fundus of patients with inflamed stomachs, as this study has shown, then a single therapy consisting of a smaller, specific combination of adhesin or receptor analogues, could be effective at treating *H. pylori* infection in both the antral and fundal regions of the stomach. Additionally, inhibitors could be carried on mini-particles such as nanogold particles to increase the density of the anti-adhesins. This is subject to a further grant application.

APPENDIX

Image Analysis Protocols

NIH IMAGE 1.62

METHOD 1: SURFACE COUNTS BY DILATION

Based on paper by Reinhard et al.[887] Also needed is the software package Adobe Photoshop. There is a free trial download on the internet of Version 6.0, the version used in the paper. B+W = black and white.

A. Process the Green (FITC) image of the microbial cells

1. **Threshold.** MENU: Options > Threshold. Adjust so all cells are thresholded.
2. **Convert to 32-Colour Image.**
   MENU: Options > Colour Tables > 32 Colours
   Save as 'Green Processed 1'
3. **Make Greyscale and Fill background in white. USE ADOBE PHOTOSHOP 5.0**
   MENU: Image > Mode > Greyscale
   Select 'Fill' tool in tool bar, select white and click in background of image which then turns white.
   Save as 'Green Processed 2'
4. **Open in NIH-Image, Threshold and binarise.**
   MENU: Options > Threshold (Set to 1.0)
   MENU: Process > Binary > Make Binary
   Save as 'Green Processed 3'

B. Process the Red (PI) image of the tissue

1. **Threshold.** MENU: Options > Threshold. Adjust so all the tissue is included in threshold.
2. **Invert, Sharpen and Binarise image – use closed binary function to fill in spaces in the image.** MENU: Edit > Invert
   MENU: Process > Sharpen
   MENU: Process > Binary (Use default count 4, Iterations 1) > Close
   Save as 'Red Processed 1'
3. **Fill in tissue in black.**
   Use paintbrush tool and click on black colour in LUT window.
   Save as 'Red Processed 1b'
4. **Dilate Red Image** so can count particles on surface of tissue section, particles outside dilated edge are regarded as background. **Dilate 10 pixels for C. albicans and 5 pixels for H. pylori (10 pixel dilation used in Reinhard's paper for H. pylori).**

MENU: Process > Binary (Set count to 1, Iterations 5 for H. pylori, or 10 for C. albicans) > Dilate. Save as 'Red Processed 2'

C. Count all cells in image

1. **Open Green Image (Green Processed 3) and Threshold.**

2. Count Cells.

MENU: Analyse > Analyse Particles (Select all options & min. size 1)

MENU: Analyse > Show Results

D. Count cells in background + surface of tissue

1. **Merge the Green Processed Image of the microbial cells, with the Red Processed Image where the tissue has been filled in.**

Open Green Processed 3 and Red Processed 1b.

MENU: Stacks > Windows to stack (use < and > keys to move between images).

MENU: Stacks > Average (of this stack)

MENU: Options > Threshold (adjust so entire image is b+w).

2. **Count cells.**

MENU: Analyse > Analyse Particles

MENU: Measure > Show Results

E. Count cells in background only

1. **Open Green processed Image and the Red Processed image which has been filled in and dilated.**

Open Green Processed 3 and Red Processed 2.

MENU: Stacks > Windows to stack (use < and > keys to move between images).

MENU: Stacks > Average (of this stack)

MENU: Options > Threshold (adjust so entire image is b+w).

2. **Count cells.**

MENU: Analyse > Analyse Particles

MENU: Measure > Show Results

F. Calculate the number of adherent cells in the image

Subtract the value obtained in E (background cell count) from the value obtained in D (background + surface cell count). This will give number of cells adhered to the surface of the tissue.

METHOD 2: SURFACE COUNTS BY AREA

A. AREA OF TISSUE PLUS ADHERENT CELLS

1. **Open Combined Red + Green image and make greyscale.**

MENU: Options > Greyscale

2. **Threshold so tissue and microbial cells are white, and background is black.**

MENU: Options > Threshold – adjust so particles correct size

3. **Make Binary.**

MENU: Process > Binary > Make Binary

4. **Fill in the tissue completely in white**

Use tools in toolbox after having selected 'white' in the LUT window with the eyedropper tool that appears once the tools for filling have been selected.

5. **Invert the Image, because the software only counts cells/areas that are black. Must threshold and binarise first.**

MENU: Options > Threshold

MENU: Process > Make Binary

MENU: Edit > Invert – The tissue has now turned black.

6. **Outline tissue+cells.**

Select 'Wand' tool and click this on the image in the black tissue. This has now become outlined.

7. **Measure area of this tissue.**

MENU: Analyse > Options (select 'Area' measurement).

MENU: Analyse > Set scale. Choose 'pixels' as units.

MENU: Analyse > Measure

MENU: Analyse > Show results.

B. AREA OF TISSUE

Open the Red Image of the tissue that has been filled in (Red Processed 1b). Follow steps 5 – 7 as outlined in section A (miss out steps 1 - 4 because the image is already filled in).

C. MEAN AREA OF A MICRIOBIAL CELL

1. **Open Green Image and make greyscale.**

MENU: Options > Greyscale

2. **Threshold (to 1.0 so cells are correct size and same size as used in methods to obtain other counts so no variation is introduced) and Binarise.**

MENU: Options > Threshold

MENU: Process > Binary > Make Binary

3. **Measure area of cells.**

Select 'Wand' tool and click on individual microbial cells. After clicking on a cell, it becomes outlined. Then can measure the area of the cell by MENU: Analyse > Options (select 'Area' as the measurement).

MENU: Analyse > Measure

MENU: Analyse > Show results

METHOD 3: SURFACE COUNTS BY DRAWING SPECIFIC REGION OF INTEREST AROUND ADHERENT CELLS

A. CREATE AN ROI AROUND ADHERENT CELLS ONLY

1. **Open green image made b+w (Green processed 3).**
2. **Open the Red+green image alongside this** – so can see which particles are on the surface so know which to include in the ROI.
3. On the green image, **draw a ROI around the cells that are adherent to tissue surface – use 'Trace region' ROI tool in toolbox.**
4. Threshold and Binarise. **May have to threshold again after binarising.**
5. Select units of measurement as Pixels.
   MENU: Analyse > Set Scale. Choose 'pixels'.
6. **Count cells.**
   MENU: Analyse > Analyse Particles
   MENU: Analyse > Measure
   MENU: Analyse > Show Results

B. MEASURE LENGTH/PERIMETER OF EPITHELIUM – the part on which the selected adherent cells were measured.

1. Open the red filled image mad b+w (Red Processed 1b).
2. Open Red+Green Image alongside this as a reference for where the epithelial edge is that the cells were counted on.
3. **Fill** in black (make straight square lines) the parts of the tissue edge not needed/used to count cells on.
4. **Threshold and binarise** – may need to re-threshold after binarising.
5. **Outline**. Click on 'Wand' tool and then click wand on black tissue.
6. **Measure.** MENU: Analyse > Options – choose length/perimeter.
   MENU: Analyse > Measure – Results are displayed in the 'Info' box.
7. Use the pointer or wand tool to rest on the image edges to get lengths of borders of image/parts of the image that were included in the perimeter measurement but want to exclude from the measurement since they were not the parts of the tissue surface that were used for the cell count. Subtract these values from the total to give the true length of surface across which the adherent cells were counted.

MEASUREMENT OF LENGTH OF EPITHELIAL SURFACE – TO CALCULATE NUMBER OF ADHERENT CELLS PER UNIT LENGTH OF TISSUE

1. Open Red (PI) Image that has been filled in black (Red Processed 1b)
2. **Threshold** so can perform measurements.
   MENU: Options > Threshold (Adjust so all tissue is filled).

3. **Choose measurements.**

MENU: Analyse > Options (select 'Perimeter/Length')

MENU: Analyse >Set scale (select 'pixels' as the units)

4. **Outline tissue.**

Select 'wand' tool in toolbox. This is the automatic outlining tool.

Click wand inside the tissue required to be measured. Tissue outline should be highlighted.

5. **Measure.**

MENU: Analyse > Measure

6. Subtract from the total perimeter value given, the length of the lines that are not wanted to be included in the measurements (i.e.) the borders of the image window. This will then give a value for the length of the tissue surface alone. These lengths to subtract can be obtained by moving the mouse pointer to the end of the lines that want to exclude. Displayed in the 'Info' box are the X-Y co-ordinates of the position of the mouse pointer. The X-Y co-ordinates represent the pixel number, thus the length (no. of pixels) of the line wanted to be excluded from the total perimeter, can be obtained. Use the 'Zoom' option (magnifying glass symbol in the toolbox) to zoom in on the image so the mouse pointer can be positioned more accurately at the edges of the lines. To 'unzoom' go to MENU: Edit > Unzoom.

IP LAB

This software cannot perform counts therefore use the 'Area Method' (find area of adherent cells and divide by mean size of one cell to obtain a cell count) to overcome this.

METHOD: SURFACE COUNTS BY AREA

A. AREA OF TISSUE+ADHERENT CELLS

1. Open the Red + Green (PI and FITC combined) image.
2. **Make Greyscale.** MENU: Enhance > Colour to Grayscale.
3. **Segment Image.** MENU: Analyse > Segmentation. Select 'White'.
4. **Fill image in black.** Double click on the 'Square' tool and select 'fill'. Select 'black' in the segment colour box. Draw squares in the image and fill inside each with black. Must fill even small dots of white with black and even the very edges of the Image window (zoom in to do this).
5. **Binarise.** Choose segment colour 'White' on the tool box. MENU: Math > Binarise.
6. **Binarise and segment again** to select the tissue.

MENU: Math > Binarise. Select 'White'.

MENU: Analyse > Segmentation. Select 'Black' tissue fills in black.

7. **Outline tissue + adherent cells.**

In toolbox: Select 'Wand' tool, select 'Segmentation tools' and select colour as 'Black'.
Click on black part of image. The cells that were attached to the surface of the epithelium have also been included in the outline.

8. **Measure area of the tissue + adherent cells (outlined part of image).**

MENU: Analyse > Set measurements. Select 'Area'.

MENU: Analyse > Measure ROI.

B. AREA OF TISSUE

Repeat steps outlined in 'A' but use the Red (PI) image.

C. MEAN AREA OF ONE CELL

1. Open the Green (FITC) Image.
2. **Make Greyscale.** MENU: Enhance > Colour to Grayscale.
3. **Segment.** MENU: Analyse > Segmentation. Select colour as 'Green'.
4. **Fill cells in white.** Zoom in using magnifying glass tool.

Choose the 'Segment Rectangle' tool.
Double click on its icon in the tool box and in the dialog box that appears select 'Pen size' as '1' and tick the 'fill' option. Click on 'ok'. Select 'White' as the colour in the tool box. Click on each pixel within each cell and they fill with white.

5. **Measure area of cells.**

Select 'Wand' tool in toolbox, select 'White' as the colour and select 'segmentation tools'.

Click wand on each cell filled in white and measure after selecting each one by doing MENU: Analyse > Measure ROI.

6. **Calculate the Mean size.** Results are displayed in 'Measurement results' window.

D. CALCULATE NUMBER OF ADHERENT CELLS

To calculate the number of adherent cells, subtract the value obtained in 'B' (area of tissue) from the value obtained in 'A' (area of tissue + adherent cells). The value that is obtained from this is the total AREA of the adherent cells. In order to obtain the NUMBER of adherent cells, divide the TOTAL AREA of adherent cells by the MEAN AREA of one cell (value obtained in 'C').

E. OBTAIN THE LENGTH OF THE EPITHELIAL SURFACE

1. **Outline the tissue** of the red image that has been filled in (Use 'Wand' tool, after selecting black as the ROI colour in the toolbox, and click on the tissue part of the image).
2. **Measure**. MENU: Analyse > Set Measurements. Select 'Area' and in 'Perimeter'. select 'pixel method'.
3. MENU: Analyse > Measure ROI. Measurement results are displayed in a separate window.
4. To subtract the lengths of the edges of the image that are not to be included in the meaurement, obtain the values by taking the x and y co-oordinates of the edges. These are

displayed in the window that appears when select MENU: Windows > Show Status. Use the pointer to obtain the position of the edges.

IMAGE PRO+

NOTE: When using processed images that have already been saved, before carrying out any functions, first the images may need to be segmented (always set segment value to 105).

METHOD 1: SURFACE COUNTS BY MERGING IMAGES

A. PROCESS THE RED IMAGE

1. Open red (PI) image.
2. **Enhance contrast so can see red more clearly.**

a) Click on 'Contrast Enhance' tool on tool bar and Increase the GAMMA contrast channel so red becomes brighter but with little increase in background noise.

b) Press 'Apply'.

3. **Make Image greyscale.** MENU: Edit > Convert to > Greyscale 8
4. **Increase contrast again** as for step 2.
5. **Threshold – makes image b+w.** MENU: Process > Threshold – set at 1.0 so black is at darkest level. Click 'Apply mask'. Then close dialog box.
6. **Fill in Image.**

a) Create AOI – select required shape on toolbar and draw on image.

b) MENU: Edit > Fill – in 'Fill' dialog box select 'black' and press 'fill'.

c) Click 'New AOI' in tool bar, then draw new AOI and fill as before. Repeat until whole tissue is filled in black.

d) Outline by hand the parts of the edge need to fill in black – Use 'Irregular AOI' tool, in the same way filling as before.

e) Fill in background in white – same method as a) – d).

B. PROCESS GREEN IMAGE

1. Increase Gamma contrast of green channel to 5.4.
2. **Segment Image.** MENU: Process > Segmentation. Set green channel to 105, choose 'Black on White'.

C. COUNT OF TOTAL CELLS IN IMAGE

1. Open Green Processed Image.
2. **Fill over any dirt.** Choose the 'Autotrace' tool from tool bar. Draw a ROI with it around the dirt and fill in the same colour as the background. MENU: Edit > Fill.

3. **Count Cells.** MENU: Measure > Count/Size. In the count/size window select the following options:

DISPLAY OPTIONS: Outline style: Outline Choose colour 'Red'.

Label Style: Object No.

Label Colour: Blue

OBJECT OPTIONS: Select: 8-Connect, Pre-Filter, Fill Holes,

Clean Borders: No borders. Click 'Ok'

4. **Select Measurements.** In Count/size window:

MENU: Measure > Select Measurements: Area and Count (adjusted). Select 'AutoDark objects' if cells are black, if cells are white, select 'Autobright objects'.

5. **Count cells.** Click 'Count' and counts are displayed.

6. **Separate clumps/clusters.** In count/size window:

MENU: Edit > Autosplit.

MENU: Edit >Watershed split.

Updated Count is automatically displayed.

D. COUNT OF CELLS IN BACKGROUND

1. Open Red filled image and the green processed image.

2. **Merge Images.** MENU: Acquire > Sequence tools > Merge

MENU: Acquire > Sequence tools > Average.

3. **Decrease gamma contrast of white channel.** Only the cells in the background remain visible.

4. **Segment this Image.**

5. **Fill over any dirt in the background.** Fill with the background colour.

6. **Count cells.** MENU: Measure > Count/size. In the Count/size window select: 'Auto bright objects'.

7. **Split clusters of cells.** In Count/size window do:

MENU: Edit > Autosplit

MENU: Edit > Watershed split. When 'Watershed split' is done, the actual tissue becomes divided up into segments. Count the number of segments this has been divided up into and subtract this value from the total, so that the value obtained only represents the number of cells rather than the tissue as well as cells.

E. COUNTS OF CELLS ON TISSUE

1. **Merge Red filled tissue image with Green processed image.**

2. **Average.**

3. **Increase gamma contrast.** Only cells on tissue remain visible.

4. **Count cells.** Select 'Auto dark objects'.

F. CALCULATE THE NUMBER OF ADHERENT CELLS

Subtract the value obtained in 'D' and 'E' (number of cells in the background and on the tissue) from the total number of cells in the image (Method 1, part 'C') and this will give the number of cells adhering to the tissue surface.

METHOD 2: SURFACE COUNTS BY AREA

A. AREA OF RED FILLED TISSUE

1. Open Red filled image made b+w.
2. **Outline tissue.** Click on 'Auto trace' tool. Click on 'Wand'. Then click the wand inside the black tissue. Tissue becomes outlined.
3. **Measure.**

   MENU: Measure > Count/size. In Count/size window do:

   MENU: Measure > Select Measurements. Choose 'Area' and click 'ok'. Choose 'Auto Dark objects'.

   Options: Label style > Measurement. Clean Borders > None.

   Then press 'Count'.
4. **View measurements.**

MENU: View > Measurement data. Area is displayed.

B. AREA OF RED+GREEN IMAGE

1. Open Red+Green image. Make greyscale.
2. **Make b+w.** Increase gamma contrast for Red channel fully. Click 'Apply'.
3. **Segment.** MENU: Process > Segmentation. Set Green channel to '105'. **Save Image.**
4. **Outline tissue and fill in black.** As described in Method 1. By hand outline any parts of tissue edge that have not been filled in, and then do 'Fill'. (select 'New AOI' on tool bar then 'Auto trace' tool. Select 'trace' rather than wand, and draw by hand an outline on the parts that have not been outlined, then fill this in).
5. **Outline the tissue, now filled in.** As described in 'A'.
6. **Measure the area.** Also described in 'A'.

C. AREA (MEAN SIZE) OF ONE MICROBIAL CELL

1. Open Green processed image (made b+w).
2. **Draw rectangular ROI** around some of the microbial cells in the image.
3. **Measure area of these cells.** MENU: Measure > Count/size. Choose 'Area' as the measurement under 'Options'.
4. **Export Data into MS Excel to calculate mean area of cells.** On 'Measurement data' window do MENU: File > DDE to Excel.
5. In MS Excel (automatically opened when did Dynamic Date Exchange, DDE), calculate the mean area of these cells.

Repeat so obtain mean area for approimately30 cells.

D. CALCULATE NUMBER OF ADHERENT CELLS

Subtract the value obtained in 'A' (area of tissue) from the value obtained in 'B' (area of tissue + adherent cells) and divide this number by the value obtained in 'C' (mean size of 1 microbial cell). This will give the number of cells adhering to the surface of the tissue.

METHOD 3: SURFACE COUNTS BY DILATION

A. TOTAL CELLS IN IMAGE

Follow the steps outlined in method 1.

B. COUNTS OF CELLS IN BACKGROUND – DILATED IMAGE

1. Open the Red filled in tissue image.
2. **Segment.** Set to 105. Tissue becomes white – need this because dilate function only dilates edges of bright objects.
3. **Fill over dirt in background.**
4. **Dilate.** MENU: Process > Filters. Select the following options: In the 'Morphological tab' for H. pylori choose 5 Passes (dilates by 5) and for C. albicans choose 10 passes (dilates by 10). Select for both '5x5 circle'.
Use 'Morphological' tab and select 'Dilate'.
5. **Merge with Green processed image.**
6. **Average.**
7. **Increase Gamma contrast.** Only cells in the background remain visible.
8. **Count cells.** Select 'Auto dark objects'.

C. COUNTS OF CELLS ON TISSUE

Use tissue count obtained in Method 1.

D. CALCULATING NUMBER OF ADHERENT CELLS

Subtract the values obtained in B (Number of cells in background) and C (Number of cells on the tissue) from the value obtained in A (total number of cells in image) and this will give the number of adherent cells (cells classed as adherent that lie within the region of the image that was dilated).

METHOD 4: SURFACE COUNTS BY DRAWING SPECIFIC REGION OF INTEREST AROUND ADHERENT CELLS

A. CREATE A ROI AROUND ADHERENT CELLS ONLY

1. Open green Image and Red+Green Image alongside it - reasons stated on other package protocols.
2. Increase Gamma contrast of Green channel (so can see green clearly) and press 'Apply'.

3. **Threshold.** MENU: Process > Segmentation. Select green channel and set so decreases the background noise. Press 'Apply Mask' and 'Close'.
4. Draw AOI around adherent cells – Use 'Irregular AOI' tool on the 'trace' option. Click with Right mouse button to end trace.
5. **Count cells.** MENU: Measure > Count/size. Choose 'Auto dark objects' because cells have become black after segmentation. Press 'Count'.
6. **Separate clusters/clumps.** On the Count/Size window do MENU: Edit > Autosplit. Then MENU: Edit > Watershed split.

B. MEASURE LENGTH/PERIMETER OF EPITHELIUM – the part on which the adherent cells were counted.
1. Outline with the 'Trace' tool the tissue part of the Red filled image.
2. Select 'perimeter' on the count/size window MENU: Measure > Select Measurements.
3. In the 'Options' section select: Borders 'none' and fill holes 'off'.
4. In the count/size window select View: Measurement data. The perimeter length is displayed (in pixels).

NOTE: The measurement doesn't include the edge of the window, only the surface of the tissue, so don't need to subtract the lengths of the unwanted areas measured from the total.

METAMORPH

The software package 'MS Photoeditor' is also needed for 3 steps of the image processing.

METHOD 1: SURFACE COUNTS BY MERGING IMAGES (NORMAL COUNTING METHOD)

A. PROCESS THE RED IMAGE
1. Open MS Photoeditor.
2. Open Red (PI) Image.
3. **Make Red brighter.** MENU: Image > Balance. Adjust the Gamma channel so red is brighter only on the PI stained tissue part of the image (not background as well).
4. **Make Greyscale.** MENU: File > Properties > Image Type. Select 'Greyscale 8-BIT' from drop-down menu.
5. **Sharpen.** MENU: Effects > Sharpen (choose strength as 6).
6. **Save.**
7. Open this Red greyscale image in Metamorph.
8. **Fill tissue in grey.** Use threshold tool on side of Image window, do Auto Threshold for light objects.

Draw ROI boxes and fill by MENU: Display >Graphics >Paint Region.

Select colour that matches e.g. Grey level 65 and 'Inside region area'. Press 'Paint'.

9. **Outline the Tissue**. MENU: Regions > Create Regions around objects. Click on black (background) part of image and then the grey tissue part of image is outlined.

10. **Fill** this all in. Click on tissue so outline becomes active and Fill as before. Fill backgound in black. Then **Threshold.** MENU: Measure > Threshold Image (adjust so all tissue is included).

NOTE: Colour combine (step of merging images used later on) does not work on binarised images, so binarise images later on.

B. PROCESS THE GREEN IMAGE

1. **Make Greyscale.** Open in MS Photoeditor and follow same steps as for Red Image but exclude step 4, because the cells are already bright enough. **Save Image.**

2. **Turn cells white** so can be counted later on (Software only counts white particles).
Click on Contrast tool on side of Image window. Select 'Auto-enhance'

3. **Threshold.** Adjust so all the cells are included.

4. **Fill in any areas of dirt/noise.** Use 'Irregular AOI' tool, click on 'trace/wand' and draw line around part of image to fill in. Double click to finish trace. Then fill in by procedure described earlier.

C. COUNT TOTAL CELLS IN IMAGE

1. Open the Green processed Image.
2. **Threshold** (set to 30) and **Binarise.**
3. **Count cells.** MENU: Apps > Count Cells.
4. In 'Count cells' dialog box click on 'configure' and select the first option.... **'counts of single objects....'** Check the 'display result imsge' box.
5. In image window, click on the colour of the cells to be counted (click on 1 cell).
6. Press 'Measure' in the count cells dialog box.
7. Result is displayed in dialog box and on screen.

D. COUNT CELLS IN BACKGROUND ONLY

1. Open the Red-grey image and threshold.
2. **Fill** in tissue in red (MENU: Graphics > PAINT REGION. Choose 'Inside region area' and 'Inside threshold') and background in black (MENU: Display > Graphics > PAINT REGION. Choose 'Outside region area' and 'Value from Variable').
3. MENU: Regions > Clear Regions and binarise. Make tissue an active ROI (MENU: Regions > Create regions around objects'. Click on the tissue part of the image). Copy the image.
4. Open Green-grey image. Make b+w (Auto-enhance contrast, threshold and binarise).
5. **Paste** ROI image of tissue onto the green (binarised) image of the cells.
6. Fill in the transferred image and 'Clear Regions'. This allows adherent cells to merge with the tissue and so a count of cells in the background only is obtained.

7. **Count cells.**

E. COUNT CELLS IN TISSUE ONLY

1. Use the red-filled image (as above; background black and tissue red).
2. **Create an ROI** around the tissue (MENU: Regions > Create Regions around objects).
3. **Fill** background in red (MENU: Display > Graphics > PAINT REGION. Choose 'Outside region area' and 'Inside Threshold') and the tissue in black (MENU: Display > Graphics > PAINT REGION. Choose 'Inside region area' and 'Value from Variable').
4. MENU: Regions > Clear regions. MENU: Regions > Create Region around objects. Click on the background (now red).
5. Select the thresholded green-grey image and click on this image. **Transfer region.** MENU: Regions > Transfer region. The region selected on the red image now becomes superimposed.
6. Don't fill in the transferred image. Make the background black and this will allow a count of cells on the tissue only.
7. Clear regions, binarise and **Count cells.**

F. CALCULATE NUMBER OF ADHERENT CELLS

Take values obtained in 'D' (number of cells in the background) and 'E' (number of cells in the tissue) and subtract these from the value obtained in 'C' (Total cells in image) to give number of adherent cells on the tissue surface.

METHOD 2: SURFACE COUNTS BY MERGING IMAGES (STANDARD AREA COUNTING METHOD)

Follow the procedures outlined in Method 1, except when it comes to counting cells, in the Count Cells dialog box click on 'Configure' and select the 2nd option, the method that counts cells using a standard area (the mean area) of a single cell, in order to divide and count the number of cells in clusters/clumps as well as single **discrete** cells. Press 'next' and enter in the 'Standard area' spin box the average size (Standard area) of a cell, calculated by the procedure outlined below.

OBTAINING THE STANDARD AREA OF A CELL

1. Open the Green Image made greyscale.
2. MENU: Apps > Count Cells > Configure > Set Counting Method as 'Use Standard area to estimate objects in a cluster' > Next
3. **Turn Cells White.** Contrast tool > Auto enhance contrast.
4. **Threshold.** Adjust so that all cells are included (MENU: Measure > Threshold Image).
5. **Binarise.**
6. Draw an ROI around lots of single cells using ROI tools.

7. **Measure.** MENU: Measure > Integrated Morphometry Anlysis (IMA). Set up the parameters for measuring as Area and Pixel area. Click in the ROI on the image so that it becomes active so the cells can be measured. Press 'Measure' in the IMA window.
8. In the IMA window click on 'display' and choose 'Summary' from the drop-down menu. This gives mean and SD of the area of cells counted. Alternatively look in the count cells window (MENU: Apps > Count cells) and the standard area of one cell has been added (Look in Configure > Set Counting method > 'Use standard Area....' of the count cells dialog box)
9. Then when doing the actual counting, **enter the Standard Area obtained.** (From the 'Measure' menu, choose 'Configure object Standards'. In the window that appears enter the mean area that was calculated, enter the value in the 'Standard Area' box. Then choose 'ok'). All clumps/clusters of cells will now be divided by this number, to give the composite number of cells in the cluster.

METHOD 3: SURFACE COUNTS BY DILATION (NORMAL COUNTING METHOD)

A. PROCESS THE RED IMAGE - DILATE

1. Open Red Greyscale filled image.
2. Fill over any dirt on the image in black.
3. **Threshold and Binarise.**
4. **Dilate.** MENU: Process > Binary. Select 'Dilate'. Choose 'Neighbourhood' as 1 and 'Repeat Count' as 10 for C. albicans and 5 for H. pylori. Press 'apply'. This dilates by 10 pixels and 5 pixels respectively.

B. COUNT TISSUE+ ADHERENT CELLS

1. **Invert** the Processed Red binarised image, so background fills in white.
2. **Create ROI of this background** – as before, MENU: Regions > Create Regions around objects. Click on this background to activate the ROI.
3. **Copy and Paste** this background onto the binarised Green greyscale Image. MENU: Regions > Transfer region. Fill the ROI in white MENU: Display > Graphics > Paint Region. Select Grey Value 1 and Paint within Region Area.
4. **Count cells**. Use Normal method, for both 5x and 10x pixel dialtion.

C. COUNT CELLS ON TISSUE

Use counts previously generated/obtained by merging filled tissue with the processed Green image (Method 1).

D. CALCULATE NUMBER OF ADHERENT CELLS

Subtract values obtained in C from values obtained in B.

METHOD 4: SURFACE COUNTS BY DILATION (STANDARD AREA COUNTING METHOD)

As for Method 3 but obtain counts using the Standard area method of counting.

METHOD 5: SURFACE COUNTS BY AREA

A. AREA OF TISSUE

1. Use tissue image that has been filled in with red.
2. Cover up any 'noise' in the background with black.
3. **Threshold** (set to 30) and **binarise** - so that the tissue becomes white.
4. **Outline the tissue.** Menu: Regions > Create regions around objects. Click in white tissue so the ROI becomes active.
5. **Measure.** MENU: Measure > Region measurements. Select 'Area' on the 'configure' tab. Click on 'measurements' tab and the measured results can be viewed.

If do MENU: Measure > Show Region Statistics, can check that the measurements are correct because the height and width of the ROI measured, is displayed.

B. AREA OF TISSUE + ADHERENT CELLS

1. Fill in the Red+Green Image.
2. Open in MS Photoeditor.

   Sharpen. Menu: Effects > Sharpen.

   Make Red clearer. MENU: Image > Balance. Increase Gamma contrast to make the red clearer so can get better outline when come to fill in the image later on.

   Make Greyscale. MENU: File > Properties > Image Type. Select 'Greyscale 8-BIT' from drop-down menu.

   Save.
3. Open this greyscale Image in Metamorph.
4. **Threshold** (set to 30). Some images,(e.g. 10^7), can't see edges very well when threshold at 30, so use Threshold tool on side of image window select 'Autothreshold for light objects'. This doesn't increase the sizes of cells/edges of tissue, so is fine to use.
5. **Binarise** and **Fill over any dirt on the image.**
6. **Create ROI around tissue and adherent cells now filled in.**

   FOLLOW ALL SAME STEPS AS FOR 'A'.

Merge the green binarised image with the red filled image by copy and paste (as in Method 3, 'B'). Measure the area of this to get the area of tissue+adherent cells.

C. CALCULATE THE NUMBER OF ADHERENT CELLS

1. Subtract the value obtained in 'A' from the value obtained in 'B' to give the **AREA** of adherent cells.
2. Divide this area by the mean area (or Standard area) of 1x microorganism (cell), to give the **NUMBER** of adherent cells.

METHOD 6: SURFACE COUNTS BY DRAWING SPECIFIC REGION OF INTEREST AROUND ADHERENT CELLS (NORMAL COUNTING METHOD)

A. CREATE AN ROI AROUND ADHERENT CELLS ONLY

1. Open the Green greyscale Image – make black and white.
2. Make pixels white so can count – click on contrast tool on side of image window and press 'auto enhance'.
3. **Threshold.** MENU: Measure > Threshold Image. Adjust so white turns red (overlay colour) but size of cells does not get larger, use magnifying glass tool to check this.
4. **Binarise.** MENU: Process > Binary. Select 'Binarise' and click 'Apply'.
5. **Draw ROI around adherent cells.** Use 'Trace Region' tool on tool bar. Refer to the Red+Green image to see which are the adherent cells to be included in the ROI.
6. **Count cells.** MENU: Apps > Count cells. Set up as usual. Click on a cell in the ROI so the ROI becomes active (flashes).
7. **Measure.** Click 'Measure'.

B. MEASURE LENGTH/PERIMETER OF EPITHELIAL SURFACE – the part on which the adherent cells were counted.

1. Open red greyscale image filled in.
2. Fill in or over parts of image tissue that don't want the length of, with boxes – calculate as for NIH-Image.
3. **Outline tissue.** MENU: Regions > Create Regions around objects. Click on tissue.
4. **Get Measurements.** MENU: Measure > Region measurements. Click on tissue to activate region.

METHOD 7: SURFACE COUNTS BY DRAWING SPECIFIC REGION OF INTEREST AROUND ADHERENT CELLS (STANDARD AREA COUNTING AREA METHOD)

As for Method 6, but obtain counts using the Standard area method of counting.

MEASUREMENT OF LENGTH OF EPITHELIAL SURFACE – TO CALCULATE NUMBER OF ADHERENT CELLS PER UNIT LENGTH OF TISSUE

1. Open Red Image.
2. Threshold (MENU: Measure > Threshold Image).
3. **Fill tissue in red.**

   Draw ROI boxes and fill by MENU: Display >Graphics >Paint Region.

   Select colour 'Red' and 'Inside region area'. Press 'Paint'.
4. **Outline the Red Image.** MENU: Regions > Create Regions around objects. Click on black (background) part of image and then the red tissue part of image is outlined.
5. **Fill** this all in. Click on tissue so outline becomes active and Fill as before.
6. Then **Threshold** and **binarise.**
7. **Measure.** MENU: Measure > Region Measurements. Select 'Include: Active Region'. Select 'Area' and 'Distance'.
8. Remove from the value obtained, the lengths of the parts of the image not wanted to be included in the measurement, i.e. the edges of the image. Do this by placing the pointer on the edge of the image and obtaining the X-Y coordinates (i.e. Pixel no./length) of the line want to subtract from the total. Zoom in to be more accurate.

References

1. Ofek,I. (1994) *Bacterial adhesion to cells and tissues.*, Chapman and Hall, New York.

2. Ofek,I., Hasty,D.L., and Sharon,N. (2003) Anti-adhesion therapy of bacterial diseases: prospects and problems. *FEMS Immunol.Med.Microbiol.* **38** (3), 181-191.

3. Kelly,C.G. and Younson,J.S. (2000) Anti-adhesive strategies in the prevention of infectious disease at mucosal surfaces. *Expert.Opin.Investig.Drugs* **9** (8), 1711-1721.

4. Murray, P. R. Medical Microbiology. Rosenthal, K. S., Kobayashi, G. S., and Pfaller, M. A. [4th Edition]. 2002. St. Louis, Missouri, USA., Mosby, Inc.

5. Wilson,M., McNab,R., and Henderson,B. (2002) *Bacterial Disease Mechanisms: An Introduction to Cellular Microbiology.*, Cambridge University Press, Cambridge, UK.

6. Bailey,A., Wadsworth,E., and Calderone,R. (1995) Adherence of Candida albicans to human buccal epithelial cells: host-induced protein synthesis and signaling events. *Infect.Immun.* **63** (2), 569-572.

7. Bernhardt,J., Herman,D., Sheridan,M., and Calderone,R. (2001) Adherence and invasion studies of Candida albicans strains, using in vitro models of esophageal candidiasis. *J.Infect.Dis.* **184** (9), 1170-1175.

8. Cossart,P. and Sansonetti,P.J. (2004) Bacterial invasion: the paradigms of enteroinvasive pathogens. *Science* **304** (5668), 242-248.

9. Cameron,C.E. (2003) Identification of a Treponema pallidum laminin-binding protein. *Infect.Immun.* **71** (5), 2525-2533.

10. Edwards,A.M., Dymock,D., Woodward,M.J., and Jenkinson,H.F. (2003) Genetic relatedness and phenotypic characteristics of Treponema associated with human periodontal tissues and ruminant foot disease. *Microbiology* **149** (Pt 5), 1083-1093.

11. Sela,M.N. (2001) Role of Treponema denticola in periodontal diseases. *Crit Rev.Oral Biol.Med.* **12** (5), 399-413.

12. Critchley,I.A. and Douglas,L.J. (1987) Role of glycosides as epithelial cell receptors for Candida albicans. *J.Gen.Microbiol.* **133** (3), 637-643.

13. Klotz,S.A., Rutten,M.J., Smith,R.L., Babcock,S.R., and Cunningham,M.D. (1993) Adherence of Candida albicans to immobilized extracellular matrix proteins is mediated by calcium-dependent surface glycoproteins. *Microb.Pathog.* **14** (2), 133-147.

14. Ringner,M., Valkonen,K.H., and Wadstrom,T. (1994) Binding of vitronectin and plasminogen to Helicobacter pylori. *FEMS Immunol.Med.Microbiol.* **9** (1), 29-34.

15. Boren,T., Falk,P., Roth,K.A., Larson,G., and Normark,S. (1993) Attachment of Helicobacter pylori to human gastric epithelium mediated by blood group antigens. *Science* **262** (5141), 1892-1895.

16. Walker,W.A. (1985) Role of the mucosal barrier in toxin/microbial attachment to the gastrointestinal tract. *Ciba Found.Symp.* **112** 34-56.

17. Lamont, J. T. Mucus: the front line of intestinal mucosal defense. Annals of the New York Academic of Science 664, 192-201. 1992.

18. Karam,S.M. (1999) Lineage commitment and maturation of epithelial cells in the gut. *Front Biosci.* **4** D286-D298.

19. Lehr,C.M., Poelma,F.G.J., Junginger,H.E., and Tukker,J.J. (1991) An estimate of the turnover time of intestinal mucus gel layer in the rat in situ loop. *Int.J.Pharm.* **70** (3), 235-240.

20. van Oss,C.J., Good,R.J., and Chaudhury,M.K. (1986) Nature of the antigen-antibody interaction. Primary and secondary bonds: optimal conditions for association and dissociation. *J.Chromatogr.* **376** 111-119.

21. Bos,R., van der Mei,H.C., and Busscher,H.J. (1999) Physico-chemistry of initial microbial adhesive interactions--its mechanisms and methods for study. *FEMS Microbiol.Rev.* **23** (2),

22. van Oss,C.J., Absolom,D.R., and Neumann,A.W. (1983) Interaction of phagocytes with other blood cells and with pathogenic and nonpathogenic microbes. *Ann.N.Y.Acad.Sci.* **416** 332-350.

23. Pethig,R. (1996) A.C. Electrokinetic Manipulation of Bioparticles. In: Coombs,R.R.H. and Robinson,D.W. (eds), pp. 153-168, Gordon & Breach Publishers, Netherlands.

24. Denyer, S. P. Mechanisms of microbial adherence. Hanlon, G. W. and Davies, M. C. In: Microbial biofilms: formation and control. 13-27. 1993. Oxford, Blackwell Scientific Publications.

25. Cotter,G. and Kavanagh,K. (2000) Adherence mechanisms of Candida albicans. *Br.J.Biomed.Sci.* **57** (3), 241-249.

26. Jones,D.S., Gorman,S.P., McCafferty,D.F., and Woolfson,A.D. (1991) The effects of three non-antibiotic, antimicrobial agents on the surface hydrophobicity of certain micro-organisms evaluated by different methods. *J Appl.Bacteriol.* **71** (3), 218-227.

27. Doyle,R.J. (2000) Contribution of the hydrophobic effect to microbial infection. *Microbes.Infect.* **2** (4), 391-400.

28. Wosten,H.A. (2001) Hydrophobins: multipurpose proteins. *Annu.Rev.Microbiol.* **55** 625-646.

29. Katsikogianni,M. and Missirlis,Y.F. (2004) Concise review of mechanisms of bacterial adhesion to biomaterials and of techniques used in estimating bacteria-material interactions. *Eur.Cell Mater.* **8** 37-57.

30. Scott,J.E. (1989) Ion binding: patterns of 'affinity' depending on types of acid groups. *Symp.Soc.Exp.Biol.* **43** 111-115.

31. Tamura,M., Hirano,Y., Koruda,K., Kuwata,F., and Hayashi,K. (2005) Effects of zinc and copper on adhesion and hemagglutination of Prevotella intermedia and Prevotella nigrescens. *Oral Microbiol.Immunol.* **20** (6), 339-343.

32. Rose,R.K., Shellis,R.P., and Lee,A.R. (1996) The role of cation bridging in microbial fluoride binding. *Caries Res.* **30** (6), 458-464.

33. Hostetter,M.K. (1994) Adhesins and ligands involved in the interaction of Candida spp. with epithelial and endothelial surfaces. *Clinical Microbiology Reviews* **7** (1), 29-42.

34. Karlsson,K.A. (1998) Meaning and therapeutic potential of microbial recognition of host glycoconjugates. *Mol.Microbiol.* **29** (1), 1-11.

35. Courtney,H.S., Hasty,D.L., and Dale,J.B. (2002) Molecular mechanisms of adhesion, colonization, and invasion of group A streptococci. *Ann.Med.* **34** (2), 77-87.

36. Jonson,A.B., Normark,S., and Rhen,M. (2005) Fimbriae, pili, flagella and bacterial virulence. *Contrib.Microbiol.* **12** 67-89.

37. Nougayrede,J.P., Fernandes,P.J., and Donnenberg,M.S. (2003) Adhesion of enteropathogenic Escherichia coli to host cells. *Cell Microbiol.* **5** (6), 359-372.

38. Chaffin,W.L., Lopez-Ribot,J.L., Casanova,M., Gozalbo,D., and Martinez,J.P. (1998) Cell wall and secreted proteins of Candida albicans: identification, function, and expression. *Microbiol.Mol.Biol.Rev.* **62** (1), 130-180.

39. Odenbreit,S., Faller,G., and Haas,R. (2002) Role of the alpAB proteins and lipopolysaccharide in adhesion of Helicobacter pylori to human gastric tissue. *Int.J.Med.Microbiol.* **292** (3-4), 247-256.

40. Tokunaga,M., Kusamichi,M., and Koike,H. (1986) Ultrastructure of outermost layer of cell wall in Candida albicans observed by rapid-freezing technique. *J.Electron Microsc.(Tokyo)* **35** (3), 237-246.

41. Tronchin,G., Bouchara,J.P., Robert,R., and Senet,J.M. (1988) Adherence of Candida albicans germ tubes to plastic: ultrastructural and molecular studies of fibrillar adhesins. *Infect.Immun.* **56** (8), 1987-1993.

42. Gozalbo,D., Roig,P., Villamon,E., and Gil,M.L. (2004) Candida and candidiasis: the cell wall as a potential molecular target for antifungal therapy. *Curr.Drug Targets.Infect Disord.* **4** (2), 117-135.

43. Smyth,C.J., Marron,M.B., Twohig,J.M., and Smith,S.G. (1996) Fimbrial adhesins: similarities and variations in structure and biogenesis. *FEMS Immunol.Med.Microbiol.* **16** (2), 127-139.

44. Taylor,R.K. (1991) Bacterial adhesion to mucosal surfaces. *J Chemother.* **3 Suppl 1** 190-195.

45. Yu,L., Lee,K.K., Ens,K., Doig,P.C., Carpenter,M.R., Staddon,W., Hodges,R.S., Paranchych,W., and Irvin,R.T. (1994) Partial characterization of a Candida albicans fimbrial adhesin. *Infect.Immun.* **62** (7), 2834-2842.

46. Masinick,S.A., Montgomery,C.P., Montgomery,P.C., and Hazlett,L.D. (1997) Secretory IgA inhibits Pseudomonas aeruginosa binding to cornea and protects against keratitis. *Invest Ophthalmol.Vis.Sci.* **38** (5), 910-918.

47. Vaerman,J.P. and Delacroix,D.L. (1984) Role of the liver in the immunobiology of IgA in animals and humans. *Contrib.Nephrol.* **40** 17-31.

48. Vaerman,J.P. (1984) [Effector mechanisms of IgA]. *Ann.Biol.Clin.(Paris)* **42** (1), 61-70.

49. Owen,P., Meehan,M., Loughry-Doherty,H., and Henderson,I. (1996) Phase-variable outer membrane proteins in Escherichia coli. *FEMS Immunol.Med.Microbiol.* **16** (2), 63-76.

50. Theiss,P. and Wise,K.S. (1997) Localized frameshift mutation generates selective, high-frequency phase variation of a surface lipoprotein encoded by a mycoplasma ABC transporter operon. *J.Bacteriol.* **179** (12), 4013-4022.

51. Lingwood,C.A., Wasfy,G., Han,H., and Huesca,M. (1993) Receptor affinity purification of a lipid-binding adhesin from Helicobacter pylori. *Infect.Immun.* **61** (6), 2474-2478.

52. Sojar,H.T. and Genco,R.J. (2005) Identification of glyceraldehyde-3-phosphate dehydrogenase of epithelial cells as a second molecule that binds to Porphyromonas gingivalis fimbriae. *FEMS Immunol.Med.Microbiol.* **45** (1), 25-30.

53. Walz,A., Odenbreit,S., Mahdavi,J., Boren,T., and Ruhl,S. (2005) Identification and characterization of binding properties of Helicobacter pylori by glycoconjugate arrays. *Glycobiology* **15** (7), 700-708.

54. Malloy,P.J., Zhao,X., Madani,N.D., and Feldman,D. (1993) Cloning and expression of the gene from Candida albicans that encodes a high-affinity corticosteroid-binding protein. *Proc.Natl.Acad.Sci.U.S.A* **90** (5), 1902-1906.

55. Powell,B.L., Drutz,D.J., Huppert,M., and Sun,S.H. (1983) Relationship of progesterone- and estradiol-binding proteins in Coccidioides immitis to coccidioidal dissemination in pregnancy. *Infect.Immun.* **40** (2), 478-485.

56. Skowronski,R. and Feldman,D. (1989) Characterization of an estrogen-binding protein in the yeast Candida albicans. *Endocrinology* **124** (4), 1965-1972.

57. Falk,P., Roth,K.A., Boren,T., Westblom,T.U., Gordon,J.I., and Normark,S. (1993) An in vitro adherence assay reveals that Helicobacter pylori exhibits cell lineage-specific tropism in the human gastric epithelium. *Proc.Natl.Acad.Sci.U.S.A* **90** (5), 2035-2039.

58. Luo,G., Niesel,D.W., Shaban,R.A., Grimm,E.A., and Klimpel,G.R. (1993) Tumor necrosis factor alpha binding to bacteria: evidence for a high-affinity receptor and alteration of bacterial virulence properties. *Infect Immun.* **61** (3), 830-835.

59. Klotz,S.A., Hein,R.C., Smith,R.L., and Rouse,J.B. (1994) The fibronectin adhesin of Candida albicans. *Infect.Immun.* **62** (10), 4679-4681.

60. Patti,J.M., Allen,B.L., McGavin,M.J., and Hook,M. (1994) MSCRAMM-mediated adherence of microorganisms to host tissues. *Annu.Rev.Microbiol.* **48** 585-617.

61. Kelly,D., Conway,S., and Aminov,R. (2005) Commensal gut bacteria: mechanisms of immune modulation. *Trends Immunol.* **26** (6), 326-333.

62. Koster,W. (2001) ABC transporter-mediated uptake of iron, siderophores, heme and vitamin B12. *Res.Microbiol.* **152** (3-4), 291-301.

63. Ekins,A., Khan,A.G., Shouldice,S.R., and Schryvers,A.B. (2004) Lactoferrin receptors in gram-negative bacteria: insights into the iron acquisition process. *Biometals* **17** (3), 235-243.

64. Christie,P.J. and Vogel,J.P. (2000) Bacterial type IV secretion: conjugation systems adapted to deliver effector molecules to host cells. *Trends Microbiol.* **8** (8), 354-360.

65. Schlumberger,M.C., Muller,A.J., Ehrbar,K., Winnen,B., Duss,I., Stecher,B., and Hardt,W.D. (2005) Real-time imaging of type III secretion: Salmonella SipA injection into host cells. *Proc.Natl.Acad.Sci.U.S.A* **102** (35), 12548-12553.

66. Islam,D., Bandholtz,L., Nilsson,J., Wigzell,H., Christensson,B., Agerberth,B., and Gudmundsson,G. (2001) Downregulation of bactericidal peptides in enteric infections: a novel immune escape mechanism with bacterial DNA as a potential regulator. *Nat.Med.* **7** (2), 180-185.

67. Collier-Hyams,L.S. and Neish,A.S. (2005) Innate immune relationship between commensal flora and the mammalian intestinal epithelium. *Cell Mol.Life Sci.* **62** (12), 1339-1348.

68. Abreu,M.T., Thomas,L.S., Arnold,E.T., Lukasek,K., Michelsen,K.S., and Arditi,M. (2003) TLR signaling at the intestinal epithelial interface. *J Endotoxin.Res.* **9** (5), 322-330.

69. Shirai,Y., Hashimoto,M., Kato,R., Kawamura,Y.I., Kirikae,T., Yano,H., Takashima,J., Kirihara,Y., Saito,Y., Fujino,M.A., and Dohi,T. (2004) Lipopolysaccharide induces CD25-positive, IL-10-producing lymphocytes without secretion of proinflammatory cytokines in the human colon: low MD-2 mRNA expression in colonic macrophages. *J Clin.Immunol.* **24** (1), 42-52.

70. Billiar,T.R., Maddaus,M.A., West,M.A., Curran,R.D., Wells,C.A., and Simmons,R.L. (1988) Intestinal gram-negative bacterial overgrowth in vivo augments the in vitro response of Kupffer cells to endotoxin. *Ann.Surg.* **208** (4), 532-540.

72. Xavier,R.J. and Podolsky,D.K. (2000) Microbiology. How to get along--friendly microbes in a hostile world. *Science* **289** (5484), 1483-1484.

73. Casadevall,A. and Pirofski,L.A. (2000) Host-pathogen interactions: basic concepts of microbial commensalism, colonization, infection, and disease. *Infect.Immun.* **68** (12), 6511-6518.

74. Caramalho,I., Lopes-Carvalho,T., Ostler,D., Zelenay,S., Haury,M., and Demengeot,J. (2003) Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. *J.Exp.Med.* **197** (4), 403-411.

75. Kelly,D. and Conway,S. (2005) Bacterial modulation of mucosal innate immunity. *Mol.Immunol.* **42** (8), 895-901.

76. Stein,M., Rappuoli,R., and Covacci,A. (2000) Tyrosine phosphorylation of the Helicobacter pylori CagA antigen after cag-driven host cell translocation. *Proc.Natl.Acad.Sci.U.S.A* **97** (3), 1263-1268.

77. Atherton,J.C., Peek,R.M., Jr., Tham,K.T., Cover,T.L., and Blaser,M.J. (1997) Clinical and pathological importance of heterogeneity in vacA, the vacuolating cytotoxin gene of Helicobacter pylori. *Gastroenterology* **112** (1), 92-99.

78. Niyonsaba,F., Hirata,M., Ogawa,H., and Nagaoka,I. (2003) Epithelial cell-derived antibacterial peptides human beta-defensins and cathelicidin: multifunctional activities on mast cells. *Curr.Drug Targets.Inflamm.Allergy* **2** (3), 224-231.

79. Buckland,A.G. and Wilton,D.C. (2000) The antibacterial properties of secreted phospholipases A(2). *Biochim.Biophys.Acta* **1488** (1-2), 71-82.

80. Kagnoff,M.F. and Eckmann,L. (1997) Epithelial cells as sensors for microbial infection. *J.Clin.Invest* **100** (1), 6-10.

81. Svanborg,C., Agace,W., Hedges,S., Linder,H., and Svensson,M. (1993) Bacterial adherence and epithelial cell cytokine production. *Zentralbl.Bakteriol.* **278** (2-3), 359-364.

82. Svanborg,C., Hedlund,M., Connell,H., Agace,W., Duan,R.D., Nilsson,A., and Wullt,B. (1996) Bacterial adherence and mucosal cytokine responses. Receptors and transmembrane signaling. *Ann.N.Y.Acad.Sci.* **797** 177-190.

83. Cinamon,G. and Alon,R. (2004) Real-time in vitro assay for studying chemoattractant-triggered leukocyte transendothelial migration under physiological flow conditions. *Methods Mol.Biol.* **239** 233-242.

84. Howard,O.M., Ben Baruch,A., and Oppenheim,J.J. (1996) Chemokines: progress toward identifying molecular targets for therapeutic agents. *Trends Biotechnol.* **14** (2), 46-51.

85. Weber,C. (2003) Novel mechanistic concepts for the control of leukocyte transmigration: specialization of integrins, chemokines, and junctional molecules. *J.Mol.Med.* **81** (1), 4-19.

86. Laudanna,C., Kim,J.Y., Constantin,G., and Butcher,E. (2002) Rapid leukocyte integrin activation by chemokines. *Immunol.Rev.* **186** 37-46.

87. Cossart,P. (2004) Bacterial invasion: a new strategy to dominate cytoskeleton plasticity. *Dev.Cell* **6** (3), 314-315.

88. Galan,J.E., Pace,J., and Hayman,M.J. (1992) Involvement of the epidermal growth factor receptor in the invasion of cultured mammalian cells by Salmonella typhimurium. *Nature* **357** (6379), 588-589.

89. Cambronne,E.D. and Schneewind,O. (2005) Bacterial invasins: molecular systems dedicated to the invasion of host tissues. *Contrib.Microbiol.* **12** 181-209.

90. Dytoc,M., Gold,B., Louie,M., Huesca,M., Fedorko,L., Crowe,S., Lingwood,C., Brunton,J., and Sherman,P. (1993) Comparison of Helicobacter pylori and attaching-effacing Escherichia coli adhesion to eukaryotic cells. *Infect.Immun.* **61** (2), 448-456.

91. Shaw,R.K., Cleary,J., Murphy,M.S., Frankel,G., and Knutton,S. (2005) Interaction of enteropathogenic Escherichia coli with human intestinal mucosa: role of effector proteins in brush border remodeling and formation of attaching and effacing lesions. *Infect.Immun.* **73** (2), 1243-1251.

92. Carvalho,H.M., Teel,L.D., Kokai-Kun,J.F., and O'brien,A.D. (2005) Antibody against the carboxyl terminus of intimin alpha reduces enteropathogenic Escherichia coli adherence to tissue culture cells and subsequent induction of actin polymerization. *Infect.Immun.* **73** (4), 2541-2546.

93. Phillips,A.D., Giron,J., Hicks,S., Dougan,G., and Frankel,G. (2000) Intimin from enteropathogenic Escherichia coli mediates remodelling of the eukaryotic cell surface. *Microbiology* **146 (Pt 6)** 1333-1344.

94. Castillo-Rojas,G., Mazari-Hiriart,M., and Lopez-Vidal,Y. (2004) [Helicobacter pylori: focus on CagA and VacA major virulence factors]. *Salud Publica Mex.* **46** (6), 538-548.

95. Goosney,D.L., de Grado,M., and Finlay,B.B. (1999) Putting E. coli on a pedestal: a unique system to study signal transduction and the actin cytoskeleton. *Trends Cell Biol.* **9** (1), 11-14.

96. Orr,J.G., Leel,V., Cameron,G.A., Marek,C.J., Haughton,E.L., Elrick,L.J., Trim,J.E., Hawksworth,G.M., Halestrap,A.P., and Wright,M.C. (2004) Mechanism of action of the antifibrogenic compound gliotoxin in rat liver cells. *Hepatology* **40** (1), 232-242.

97. Shah,D.T., Jackman,S., Engle,J., and Larsen,B. (1998) Effect of gliotoxin on human polymorphonuclear neutrophils. *Infect.Dis.Obstet.Gynecol.* **6** (4), 168-175.

98. Shah,D.T., Glover,D.D., and Larsen,B. (1995) In situ mycotoxin production by Candida albicans in women with vaginitis. *Gynecol.Obstet.Invest* **39** (1), 67-69.

99. Waring,P., Khan,T., and Sjaarda,A. (1997) Apoptosis induced by gliotoxin is preceded by phosphorylation of histone H3 and enhanced sensitivity of chromatin to nuclease digestion. *J.Biol.Chem.* **272** (29), 17929-17936.

100. Glee,P.M., Cutler,J.E., Benson,E.E., Bargatze,R.F., and Hazen,K.C. (2001) Inhibition of hydrophobic protein-mediated Candida albicans attachment to endothelial cells during physiologic shear flow. *Infect.Immun.* **69** (5), 2815-2820.

101. Hazen,B.W. and Hazen,K.C. (1989) Isolation of hydrophobic and hydrophilic variants of Candida albicans. *FEMS Microbiol.Lett.* **48** (2), 167-171.

102. Hazen,K.C. (1989) Participation of yeast cell surface hydrophobicity in adherence of Candida albicans to human epithelial cells. *Infect.Immun.* **57** (7), 1894-1900.

103. Hazen,K.C., Wu,J.G., and Masuoka,J. (2001) Comparison of the hydrophobic properties of Candida albicans and Candida dubliniensis. *Infect.Immun.* **69** (2), 779-786.

104. Merkel,G.J. (1992) Effects of culture conditions on the in vitro infection of fibroblasts by Candida albicans. *Can.J.Microbiol.* **38** (2), 135-142.

105. McCourtie,J. and Douglas,L.J. (1984) Relationship between cell surface composition, adherence, and virulence of Candida albicans. *Infect.Immun.* **45** (1), 6-12.
Ref ID: 416

106. Mack,D.R., Michail,S., Wei,S., McDougall,L., and Hollingsworth,M.A. (1999) Probiotics inhibit enteropathogenic E. coli adherence in vitro by inducing intestinal mucin gene expression. *Am.J.Physiol* **276** (4 Pt 1), G941-G950.

107. Bibel,D.J., Aly,R., Bayles,C., Strauss,W.G., Shinefield,H.R., and Maibach,H.I. (1983) Competitive adherence as a mechanism of bacterial interference. *Can.J.Microbiol.* **29** (6), 700-703.

108. Reid,G., Howard,J., and Gan,B.S. (2001) Can bacterial interference prevent infection? *Trends Microbiol.* **9** (9), 424-428.

109. Sullivan,A. and Nord,C.E. (2002) The place of probiotics in human intestinal infections. *Int.J.Antimicrob.Agents* **20** (5), 313-319.

110. Sullivan,A. and Nord,C.E. (2002) Probiotics in human infections. *J.Antimicrob.Chemother.* **50** (5), 625-627.

111. Lievin,V., Peiffer,I., Hudault,S., Rochat,F., Brassart,D., Neeser,J.R., and Servin,A.L. (2000) Bifidobacterium strains from resident infant human gastrointestinal microflora exert antimicrobial activity. *Gut* **47** (5), 646-652.

112. Bagg,J. and Silverwood,R.W. (1986) Coagglutination reactions between Candida albicans and oral bacteria. *J Med.Microbiol.* **22** (2), 165-169.

113. Jenkinson,H.F., Lala,H.C., and Shepherd,M.G. (1990) Coaggregation of Streptococcus sanguis and other streptococci with Candida albicans. *Infect Immun.* **58** (5), 1429-1436.

114. Creeth,J.M. (1978) Constituents of mucus and their separation. *Br.Med.Bull.* **34** (1), 17-24.

115. Wu,A.M., Csako,G., and Herp,A. (1994) Structure, biosynthesis, and function of salivary mucins. *Mol.Cell Biochem.* **137** (1), 39-55.

116. Roberts, M. Biology principles and processes. Reiss, M. and Monger, G. 1993. UK, Nelson.
Ref Type: Generic

117. Acheson,D.W. and Luccioli,S. (2004) Microbial-gut interactions in health and disease. Mucosal immune responses. *Best.Pract.Res.Clin.Gastroenterol.* **18** (2), 387-404.

118. Mardh,P.A. (1991) The vaginal ecosystem. *Am.J.Obstet.Gynecol.* **165** (4 Pt 2), 1163-1168.

119. Boris,S. and Barb,C. (2000) Role played by lactobacilli in controlling the population of vaginal pathogens. *Microbes.Infect* **2** (5), 543-546.

120. Brockhausen,I. (2004) Intestinal candyfloss. *Biochem.J.* **384** (Pt 2), e3-e5.

121. Jacquot,J., Hayem,A., and Galabert,C. (1992) Functions of proteins and lipids in airway secretions. *Eur.Respir.J.* **5** (3), 343-358.

122. Pommerenke,W.T. and Taylor,P.W., Jr. (1953) Antibacterial properties of vaginal and cervical secretions and amniotic fluid. *Ann.Ostet.Ginecol.* **75** (6), 891-899.

123. Salathe,M., Forteza,R., and Conner,G.E. (2002) Post-secretory fate of host defence components in mucus. *Novartis.Found.Symp.* **248** 20-26.

124. Gerson,C., Sabater,J., Scuri,M., Torbati,A., Coffey,R., Abraham,J.W., Lauredo,I., Forteza,R., Wanner,A., Salathe,M., Abraham,W.M., and Conner,G.E. (2000) The lactoperoxidase system functions in bacterial clearance of airways. *Am.J.Respir.Cell Mol.Biol.* **22** (6), 665-671.

125. Holtje,J.V. (1996) Lytic transglycosylases. *EXS* **75** 425-429.

126. Takada,K., Ohno,N., and Yadomae,T. (1994) Binding of lysozyme to lipopolysaccharide suppresses tumor necrosis factor production in vivo. *Infect.Immun.* **62** (4), 1171-1175.

127. Takada,K., Ohno,N., and Yadomae,T. (1994) Lysozyme regulates LPS-induced interleukin-6 release in mice. *Circ.Shock* **44** (4), 169-174.

128. Leitch,E.C. and Willcox,M.D. (1998) Synergic antistaphylococcal properties of lactoferrin and lysozyme. *J.Med.Microbiol.* **47** (9), 837-842.

129. Sanchez,L., Calvo,M., and Brock,J.H. (1992) Biological role of lactoferrin. *Arch.Dis.Child* **67** (5), 657-661.

130. Masson,P.L., Heremans,J.F., and Schonne,E. (1969) Lactoferrin, an iron-binding protein in neutrophilic leukocytes. *J.Exp.Med.* **130** (3), 643-658.

131. Baveye,S., Elass,E., Mazurier,J., and Legrand,D. (2000) Lactoferrin inhibits the binding of lipopolysaccharides to L-selectin and subsequent production of reactive oxygen species by neutrophils. *FEBS Lett.* **469** (1), 5-8.

132. Baveye,S., Elass,E., Fernig,D.G., Blanquart,C., Mazurier,J., and Legrand,D. (2000) Human lactoferrin interacts with soluble CD14 and inhibits expression of endothelial adhesion molecules, E-selectin and ICAM-1, induced by the CD14-lipopolysaccharide complex. *Infect.Immun.* **68** (12), 6519-6525.

133. Ochoa,T.J., Noguera-Obenza,M., and Cleary,T.G. (2004) Lactoferrin blocks the initial host cell attachment mechanism of Enteropathogenic E. coli (EPEC). *Adv.Exp.Med.Biol.* **554** 463-466.

134. Ellison,R.T., III and Giehl,T.J. (1991) Killing of gram-negative bacteria by lactoferrin and lysozyme. *J.Clin.Invest* **88** (4), 1080-1091.

135. Willer,E.M., Lima,R.L., and Giugliano,L.G. (2004) In vitro adhesion and invasion inhibition of Shigella dysenteriae, Shigella flexneri and Shigella sonnei clinical strains by human milk proteins. *BMC.Microbiol.* **4** 18.

136. Sallmann,F.R., Baveye-Descamps,S., Pattus,F., Salmon,V., Branza,N., Spik,G., and Legrand,D. (1999) Porins OmpC and PhoE of Escherichia coli as specific cell-surface targets of human lactoferrin. Binding characteristics and biological effects. *J Biol.Chem.* **274** (23), 16107-16114.

137. Wakabayashi,H., Takase,M., and Tomita,M. (2003) Lactoferricin derived from milk protein lactoferrin. *Curr.Pharm.Des* **9** (16), 1277-1287.

138. Thomas,E. (1991) *Lactoperoxidase: structure and catalytic properties.Peroxidases in Chemistry and Biology.* pp. 123-142, CRC Press, Boca Raton, Florida, USA.

139. Mickelson,M.N. (1979) Antibacterial action of lactoperoxidase-thiocyanate-hydrogen peroxide on Streptococcus agalactiae. *Appl.Environ.Microbiol.* **38** (5), 821-826.

140. Roger,V., Tenovuo,J., Lenander-Lumikari,M., Soderling,E., and Vilja,P. (1994) Lysozyme and lactoperoxidase inhibit the adherence of Streptococcus mutans NCTC 10449 (serotype c) to saliva-treated hydroxyapatite in vitro. *Caries Res.* **28** (6), 421-428.

141. Reiter,B., Marshall,V.M., BjorckL, and Rosen,C.G. (1976) Nonspecific bactericidal activity of the lactoperoxidases-thiocyanate-hydrogen peroxide system of milk against Escherichia coli and some gram-negative pathogens. *Infect.Immun.* **13** (3), 800-807.
Ref ID: 445

142. Bjorck,L., Rosen,C., Marshall,V., and Reiter,B. (1975) Antibacterial activity of the lactoperoxidase system in milk against pseudomonads and other gram-negative bacteria. *Appl.Microbiol.* **30** (2), 199-204.

143. Johansen,C., Falholt,P., and Gram,L. (1997) Enzymatic removal and disinfection of bacterial biofilms. *Appl.Environ.Microbiol.* **63** (9), 3724-3728.

144. Buckland,A.G., Heeley,E.L., and Wilton,D.C. (2000) Bacterial cell membrane hydrolysis by secreted phospholipases A(2): a major physiological role of human group IIa sPLA(2) involving both bacterial cell wall penetration and interfacial catalysis. *Biochim.Biophys.Acta* **1484** (2-3), 195-206.

145. Beers,S.A., Buckland,A.G., Koduri,R.S., Cho,W., Gelb,M.H., and Wilton,D.C. (2002) The antibacterial properties of secreted phospholipases A2: a major physiological role for the group IIA enzyme that depends on the very high pI of the enzyme to allow penetration of the bacterial cell wall. *J.Biol.Chem.* **277** (3), 1788-1793.

146. da Silva,M.C., Zahm,J.M., Gras,D., Bajolet,O., Abely,M., Hinnrasky,J., Milliot,M., de Assis,M.C., Hologne,C., Bonnet,N., Merten,M., Plotkowski,M.C., and Puchelle,E. (2004) Dynamic interaction between airway epithelial cells and Staphylococcus aureus. *Am.J.Physiol Lung Cell Mol.Physiol* **287** (3), L543-L551.

147. Doumas,S., Kolokotronis,A., and Stefanopoulos,P. (2005) Anti-inflammatory and antimicrobial roles of secretory leukocyte protease inhibitor. *Infect.Immun.* **73** (3), 1271-1274.

148. Yang,J., Zhu,J., Sun,D., and Ding,A. (2005) Suppression of macrophage responses to bacterial lipopolysaccharide (LPS) by secretory leukocyte protease inhibitor (SLPI) is independent of its anti-protease function. *Biochim.Biophys.Acta* **1745** (3), 310-317.

149. Fahey,J.V. and Wira,C.R. (2002) Effect of menstrual status on antibacterial activity and secretory leukocyte protease inhibitor production by human uterine epithelial cells in culture. *J.Infect.Dis.* **185** (11), 1606-1613.

150. Tomee,J.F., Hiemstra,P.S., Heinzel-Wieland,R., and Kauffman,H.F. (1997) Antileukoprotease: an endogenous protein in the innate mucosal defense against fungi. *J.Infect.Dis.* **176** (3), 740-747.

151. Tomee,J.F., Koeter,G.H., Hiemstra,P.S., and Kauffman,H.F. (1998) Secretory leukoprotease inhibitor: a native antimicrobial protein presenting a new therapeutic option? *Thorax* **53** (2), 114-116.

152. Longman,R.J., Douthwaite,J., Sylvester,P.A., Poulsom,R., Corfield,A.P., Thomas,M.G., and Wright,N.A. (2000) Coordinated localisation of mucins and trefoil peptides in the ulcer associated cell lineage and the gastrointestinal mucosa. *Gut* **47** (6), 792-800.

153. Kindon,H., Pothoulakis,C., Thim,L., Lynch-Devaney,K., and Podolsky,D.K. (1995) Trefoil peptide protection of intestinal epithelial barrier function: cooperative interaction with mucin glycoprotein. *Gastroenterology* **109** (2), 516-523.

154. Thim,L. (1997) Trefoil peptides: from structure to function. *Cell Mol.Life Sci.* **53** (11-12), 888-903.

155. Worthley,D.L., Bardy,P.G., and Mullighan,C.G. (2005) Mannose-binding lectin: biology and clinical implications. *Intern.Med.J* **35** (9), 548-555.

156. Kim,Y.S., Ryu,J.H., Han,S.J., Choi,K.H., Nam,K.B., Jang,I.H., Lemaitre,B., Brey,P.T., and Lee,W.J. (2000) Gram-negative bacteria-binding protein, a pattern recognition receptor for lipopolysaccharide and beta-1,3-glucan that mediates the signaling for the induction of innate immune genes in Drosophila melanogaster cells. *J.Biol.Chem.* **275** (42), 32721-32727.

157. Palaniyar,N., Nadesalingam,J., and Reid,K.B. (2002) Pulmonary innate immune proteins and receptors that interact with gram-positive bacterial ligands. *Immunobiology* **205** (4-5), 575-594.

158. Du Clos,T.W. (1996) The interaction of C-reactive protein and serum amyloid P component with nuclear antigens. *Mol.Biol.Rep.* **23** (3-4), 253-260.

159. Basset,C., Holton,J., O'Mahony,R., and Roitt,I. (2003) Innate immunity and pathogen-host interaction. *Vaccine* **21 Suppl 2** S12-S23.

160. Vreugdenhil,A.C., Dentener,M.A., Snoek,A.M., Greve,J.W., and Buurman,W.A. (1999) Lipopolysaccharide binding protein and serum amyloid A secretion by human intestinal epithelial cells during the acute phase response. *J.Immunol.* **163** (5), 2792-2798.

161. Green,P.J., Feizi,T., Stoll,M.S., Thiel,S., Prescott,A., and McConville,M.J. (1994) Recognition of the major cell surface glycoconjugates of Leishmania parasites by the human serum mannan-binding protein. *Mol.Biochem.Parasitol.* **66** (2), 319-328.

162. Madan,T., Kishore,U., Singh,M., Strong,P., Hussain,E.M., Reid,K.B., and Sarma,P.U. (2001) Protective role of lung surfactant protein D in a murine model of invasive pulmonary aspergillosis. *Infect.Immun.* **69** (4), 2728-2731.

163. Hickling,T.P., Bright,H., Wing,K., Gower,D., Martin,S.L., Sim,R.B., and Malhotra,R. (1999) A recombinant trimeric surfactant protein D carbohydrate recognition domain inhibits respiratory syncytial virus infection in vitro and in vivo. *Eur.J.Immunol.* **29** (11), 3478-3484.

164. Epstein,J., Eichbaum,Q., Sheriff,S., and Ezekowitz,R.A. (1996) The collectins in innate immunity. *Curr.Opin.Immunol.* **8** (1), 29-35.

165. Wright,J.R. (1997) Immunomodulatory functions of surfactant. *Physiol Rev.* **77** (4), 931-962.

166. Hartshorn,K.L., White,M.R., and Crouch,E.C. (2002) Contributions of the N- and C-terminal domains of surfactant protein d to the binding, aggregation, and phagocytic uptake of bacteria. *Infect.Immun.* **70** (11), 6129-6139.

167. Pikaar,J.C., Voorhout,W.F., van Golde,L.M., Verhoef,J., Van Strijp,J.A., and van Iwaarden,J.F. (1995) Opsonic activities of surfactant proteins A and D in phagocytosis of gram-negative bacteria by alveolar macrophages. *J.Infect.Dis.* **172** (2), 481-489.

168. Borron,P., McIntosh,J.C., Korfhagen,T.R., Whitsett,J.A., Taylor,J., and Wright,J.R. (2000) Surfactant-associated protein A inhibits LPS-induced cytokine and nitric oxide production in vivo. *Am.J Physiol Lung Cell Mol.Physiol* **278** (4), L840-L847.

169. Kunisawa,J., Fukuyama,S., and Kiyono,H. (2005) Mucosa-associated lymphoid tissues in the aerodigestive tract: their shared and divergent traits and their importance to the orchestration of the mucosal immune system. *Curr.Mol.Med.* **5** (6), 557-572.

170. Fluckiger,U., Jones,K.F., and Fischetti,V.A. (1998) Immunoglobulins to group A streptococcal surface molecules decrease adherence to and invasion of human pharyngeal cells. *Infect.Immun.* **66** (3), 974-979.

171. Fernandes,R.M., Carbonare,S.B., Carneiro-Sampaio,M.M., and Trabulsi,L.R. (2001) Inhibition of enteroaggregative Escherichia coli adhesion to HEp-2 cells by secretory immunoglobulin A from human colostrum. *Pediatr.Infect.Dis.J.* **20** (7), 672-678.

172. Rojas,R. and Apodaca,G. (2002) Immunoglobulin transport across polarized epithelial cells. *Nat.Rev.Mol.Cell Biol.* **3** (12), 944-955.
Ref ID: 480

173. Asahi-Ozaki,Y., Yoshikawa,T., Iwakura,Y., Suzuki,Y., Tamura,S., Kurata,T., and Sata,T. (2004) Secretory IgA antibodies provide cross-protection against infection with different strains of influenza B virus. *J Med.Virol.* **74** (2), 328-335.

174. Tamura,S. and Kurata,T. (2004) Defense mechanisms against influenza virus infection in the respiratory tract mucosa. *Jpn.J Infect Dis* **57** (6), 236-247.

175. Mahida,Y.R. and Cunliffe,R.N. (2004) Defensins and mucosal protection. *Novartis.Found.Symp.* **263** 71-77.

176. Ouellette,A.J. (1999) IV. Paneth cell antimicrobial peptides and the biology of the mucosal barrier. *Am.J.Physiol* **277** (2 Pt 1), G257-G261.

177. Heller,W.T., Waring,A.J., Lehrer,R.I., Harroun,T.A., Weiss,T.M., Yang,L., and Huang,H.W. (2000) Membrane thinning effect of the beta-sheet antimicrobial protegrin. *Biochemistry* **39** (1), 139-145.

178. Gazit,E., Boman,A., Boman,H.G., and Shai,Y. (1995) Interaction of the mammalian antibacterial peptide cecropin P1 with phospholipid vesicles. *Biochemistry* **34** (36), 11479-11488.

179. Hoover,D.M., Rajashankar,K.R., Blumenthal,R., Puri,A., Oppenheim,J.J., Chertov,O., and Lubkowski,J. (2000) The structure of human beta-defensin-2 shows evidence of higher order oligomerization. *J.Biol.Chem.* **275** (42), 32911-32918.

180. Hoover,D.M., Chertov,O., and Lubkowski,J. (2001) The structure of human beta-defensin-1: new insights into structural properties of beta-defensins. *J.Biol.Chem.* **276** (42), 39021-39026.

181. Gropp,R., Frye,M., Wagner,T.O., and Bargon,J. (1999) Epithelial defensins impair adenoviral infection: implication for adenovirus-mediated gene therapy. *Hum.Gene Ther.* **10** (6), 957-964.

182. Lengyel,G. and Zsolt,T. (2005) [Failure in the eradication of Helicobacter pylori: the role of antibiotic resistance]. *Orv.Hetil.* **146** (18), 839-842.

183. Klepser,M.E. (2001) Antifungal resistance among Candida species. *Pharmacotherapy* **21** (8 Pt 2), 124S-132S.

184. Akins,R.A. (2005) An update on antifungal targets and mechanisms of resistance in Candida albicans. *Med.Mycol.* **43** (4), 285-318.

185. Deva,R., Ciccoli,R., Schewe,T., Kock,J.L., and Nigam,S. (2000) Arachidonic acid stimulates cell growth and forms a novel oxygenated metabolite in Candida albicans. *Biochim.Biophys.Acta* **1486** (2-3), 299-311.

186. Datta,A., Ganesan,K., and Natarajan,K. (1989) Current trends in Candida albicans research. *Adv.Microb.Physiol* **30** 53-88.

187. Brown,A.J. and Gow,N.A. (1999) Regulatory networks controlling Candida albicans morphogenesis. *Trends Microbiol.* **7** (8), 333-338.

188. Fidel,P.L., Jr., Vazquez,J.A., and Sobel,J.D. (1999) Candida glabrata: review of epidemiology, pathogenesis, and clinical disease with comparison to C. albicans. *Clinical Microbiology Reviews* **12** (1), 80-96.

189. Hay,R.J. (1999) The management of superficial candidiasis. *J Am.Acad.Dermatol.* **40** (6 Pt 2), S35-S42.

190. Kunzelmann,V., Tietz,H.J., Rossner,D., Czaika,V., Hopp,M., Schmalreck,A., and Sterry,W. (1996) [Prerequisites for effective therapy of chronic recurrent vaginal candidiasis]. *Mycoses* **39 Suppl 1** 65-72.

191. Segal,E. (2005) Candida, still number one--what do we know and where are we going from there? *Mycoses* **48 Suppl 1** 3-11.

192. Liebowitz,L.D., Ashbee,H.R., Evans,E.G., Chong,Y., Mallatova,N., Zaidi,M., and Gibbs,D. (2001) A two year global evaluation of the susceptibility of Candida species to fluconazole by disk diffusion. *Diagn.Microbiol.Infect.Dis.* **40** (1-2), 27-33.

193. Knoke,M. (1999) [Gastrointestinal microecology of humans and Candida]. *Mycoses* **42 Suppl 1** 30-34.

194. Bernhardt,H. and Knoke,M. (1997) Mycological aspects of gastrointestinal microflora. *Scand.J.Gastroenterol.Suppl* **222** 102-106.

195. Cohen,R., Roth,F.J., Delgado,E., Ahearn,D.G., and Kalser,M.H. (1969) Fungal flora of the normal human small and large intestine. *N.Engl.J.Med.* **280** (12), 638-641.

196. Maksymiuk,A.W., Thongprasert,S., Hopfer,R., Luna,M., Fainstein,V., and Bodey,G.P. (1984) Systemic candidiasis in cancer patients. *Am.J.Med.* **77** (4D), 20-27.

197. Meunier,F. (1987) Prevention and mycoses in immunocompromised patients. *Rev.Infect.Dis.* **9** (2), 408-416.

198. Antrum,J. (1996) Meeting the challenge of systemic fungal infections in cancer: nursing implications. *Eur.J.Haematol.Suppl* **57** 7-11.

199. Lew,M.A. (1989) Diagnosis of systemic Candida infections. *Annu.Rev.Med.* **40** 87-97.

200. Bow,E.J. (1998) Invasive fungal infections in patients receiving intensive cytotoxic therapy for cancer. *Br.J.Haematol.* **101 Suppl 1** 1-4.

201. De Pauw,B.E. and Meis,J.F. (1998) Progress in fighting systemic fungal infections in haematological neoplasia. *Support.Care Cancer* **6** (1), 31-38.

202. Rippon,J.W. (1988) *Medical Mycology: The Pathogenic Fungi and the Pathogenic Actinomycetes.*, W.B.Saunders Company, Harcourt Brace Jovanovich, Inc., Philadelphia, USA.

203. Garber,G. (2001) An overview of fungal infections. *Drugs* **61 Suppl 1** 1-12.

204. Wilson,C. (2005) Recurrent vulvovaginitis candidiasis; an overview of traditional and alternative therapies. *Adv.Nurse Pract.* **13** (5), 24-29.

205. Mardh,P.A., Rodrigues,A.G., Genc,M., Novikova,N., Martinez-de-Oliveira,J., and Guaschino,S. (2002) Facts and myths on recurrent vulvovaginal candidosis--a review on epidemiology, clinical manifestations, diagnosis, pathogenesis and therapy. *Int.J.STD AIDS* **13** (8), 522-539.

206. Lopes Consolaro,M.E., Aline,A.T., Shizue,Y.C., Mazucheli,J., Peralta,R.M., and Estivalet Svidzinski,T.I. (2004) Correlation of Candida species and symptoms among patients with vulvovaginal candidiasis in Maringa, Parana, Brazil. *Rev.Iberoam.Micol.* **21** (4), 202-205.

207. Carr,P.L., Felsenstein,D., and Friedman,R.H. (1998) Evaluation and management of vaginitis. *J Gen.Intern.Med.* **13** (5), 335-346.

208. Heidrich,F.E., Berg,A.O., and Bergman,J.J. (1984) Clothing factors and vaginitis. *J Fam.Pract.* **19** (4), 491-494.

209. Sobel,J.D. (1998) Vulvovaginitis. When Candida becomes a problem. *Dermatol.Clin.* **16** (4), 763-8, xii.

210. Eckert,L.O., Hawes,S.E., Stevens,C.E., Koutsky,L.A., Eschenbach,D.A., and Holmes,K.K. (1998) Vulvovaginal candidiasis: clinical manifestations, risk factors, management algorithm. *Obstet.Gynecol.* **92** (5), 757-765.

211. Wilson,R.M. and Reeves,W.G. (1986) Neutrophil phagocytosis and killing in insulin-dependent diabetes. *Clin.Exp.Immunol.* **63** (2), 478-484.

212. Fong,I.W. (1994) The rectal carriage of yeast in patients with vaginal candidiasis. *Clin.Invest Med.* **17** (5), 426-431.

213. Schaller,M., Borelli,C., Korting,H.C., and Hube,B. (2005) Hydrolytic enzymes as virulence factors of Candida albicans. *Mycoses* **48** (6), 365-377.

214. Stehr,F., Felk,A., Kretschmar,M., Schaller,M., Schafer,W., and Hube,B. (2000) [Extracellular hydrolytic enzymes and their relevance during Candida albicans infections.]. *Mycoses* **43 Suppl 2** 17-21.

215. Gow,N.A., Perera,T.H., Sherwood-Higham,J., Gooday,G.W., Gregory,D.W., and Marshall,D. (1994) Investigation of touch-sensitive responses by hyphae of the human pathogenic fungus Candida albicans. *Scanning Microsc.* **8** (3), 705-710.

216. Klotz,S.A. (1994) Plasma and extracellular matrix proteins mediate in the fate of Candida albicans in the human host. *Med.Hypotheses* **42** (5), 328-334.

217. Robert,R., Mahaza,C., Miegeville,M., Ponton,J., Marot-Leblond,A., and Senet,J.M. (1996) Binding of resting platelets to Candida albicans germ tubes. *Infect Immun.* **64** (9), 3752-3757.

218. Manns,J.M., Mosser,D.M., and Buckley,H.R. (1994) Production of a hemolytic factor by Candida albicans. *Infect Immun.* **62** (11), 5154-5156.

219. Casadevall,A. and Pirofski,L. (2001) Host-pathogen interactions: the attributes of virulence. *J.Infect.Dis.* **184** (3), 337-344.

220. Furman,R.M. and Ahearn,D.G. (1983) Candida ciferrii and Candida chiropterorum isolated from clinical specimens. *J.Clin.Microbiol.* **18** (5), 1252-1255.

221. Odds,F.C., Gow,N.A., and Brown,A.J. (2001) Fungal virulence studies come of age. *Genome Biol.* **2** (3), REVIEWS1009.

222. Staab,J.F., Bradway,S.D., Fidel,P.L., and Sundstrom,P. (1999) Adhesive and mammalian transglutaminase substrate properties of Candida albicans Hwp1. *Science* **283** (5407), 1535-1538.

223. Kinneberg,K.M., Bendel,C.M., Jechorek,R.P., Cebelinski,E.A., Gale,C.A., Berman,J.G., Erlandsen,S.L., Hostetter,M.K., and Wells,C.L. (1999) Effect of INT1 gene on Candida albicans murine intestinal colonization. *J.Surg.Res.* **87** (2), 245-251.

224. Buurman,E.T., Westwater,C., Hube,B., Brown,A.J., Odds,F.C., and Gow,N.A. (1998) Molecular analysis of CaMnt1p, a mannosyl transferase important for adhesion and virulence of Candida albicans. *Proc.Natl.Acad.Sci.U.S.A* **95** (13), 7670-7675.

225. Calderone,R.A. and Braun,P.C. (1991) Adherence and receptor relationships of Candida albicans. *Microbiol.Rev.* **55** (1), 1-20.

226. Cutler,J.E. (1991) Putative virulence factors of Candida albicans. *Annu.Rev.Microbiol.* **45** 187-218.

227. Franzke,S., Calderone,R.A., and Schaller,K. (1993) Isolation of avirulent clones of Candida albicans with reduced ability to recognize the CR2 ligand C3d. *Infect.Immun.* **61** (6), 2662-2669.

228. Bruckmann,A., Kunkel,W., Hartl,A., Wetzker,R., and Eck,R. (2000) A phosphatidylinositol 3-kinase of Candida albicans influences adhesion, filamentous growth and virulence. *Microbiology* **146 (Pt 11)** 2755-2764.

229. Lopez-Ribot,J.L. and Chaffin,W.L. (1994) Binding of the extracellular matrix component entactin to Candida albicans. *Infect.Immun.* **62** (10), 4564-4571.

230. Meri,T., Blom,A.M., Hartmann,A., Lenk,D., Meri,S., and Zipfel,P.F. (2004) The hyphal and yeast forms of Candida albicans bind the complement regulator C4b-binding protein. *Infect.Immun.* **72** (11), 6633-6641.

231. Saporito-Irwin,S.M., Birse,C.E., Sypherd,P.S., and Fonzi,W.A. (1995) PHR1, a pH-regulated gene of Candida albicans, is required for morphogenesis. *Mol.Cell Biol.* **15** (2), 601-613.

232. Ghannoum,M.A., Spellberg,B., Saporito-Irwin,S.M., and Fonzi,W.A. (1995) Reduced virulence of Candida albicans PHR1 mutants. *Infect.Immun.* **63** (11), 4528-4530.

233. Filler,S.G., Swerdloff,J.N., Hobbs,C., and Luckett,P.M. (1995) Penetration and damage of endothelial cells by Candida albicans. *Infect Immun.* **63** (3), 976-983.

234. Hausauer,D.L., Gerami-Nejad,M., Kistler-Anderson,C., and Gale,C.A. (2005) Hyphal guidance and invasive growth in Candida albicans require the Ras-like GTPase Rsr1p and its GTPase-activating protein Bud2p. *Eukaryot.Cell* **4** (7), 1273-1286.

235. Phan,Q.T., Belanger,P.H., and Filler,S.G. (2000) Role of hyphal formation in interactions of Candida albicans with endothelial cells. *Infect.Immun.* **68** (6), 3485-3490.

236. Warenda,A.J., Kauffman,S., Sherrill,T.P., Becker,J.M., and Konopka,J.B. (2003) Candida albicans septin mutants are defective for invasive growth and virulence. *Infect.Immun.* **71** (7), 4045-4051.

237. Rocha,C.R., Schroppel,K., Harcus,D., Marcil,A., Dignard,D., Taylor,B.N., Thomas,D.Y., Whiteway,M., and Leberer,E. (2001) Signaling through adenylyl cyclase is essential for hyphal growth and virulence in the pathogenic fungus Candida albicans. *Mol.Biol.Cell* **12** (11), 3631-3643.

238. Lo,H.J., Kohler,J.R., DiDomenico,B., Loebenberg,D., Cacciapuoti,A., and Fink,G.R. (1997) Nonfilamentous C. albicans mutants are avirulent. *Cell* **90** (5), 939-949.

239. Chen,J., Zhou,S., Wang,Q., Chen,X., Pan,T., and Liu,H. (2000) Crk1, a novel Cdc2-related protein kinase, is required for hyphal development and virulence in Candida albicans. *Mol.Cell Biol.* **20** (23), 8696-8708.

240. Gale,C.A., Bendel,C.M., McClellan,M., Hauser,M., Becker,J.M., Berman,J., and Hostetter,M.K. (1998) Linkage of adhesion, filamentous growth, and virulence in Candida albicans to a single gene, INT1. *Science* **279** (5355), 1355-1358.

241. Riggle,P.J., Andrutis,K.A., Chen,X., Tzipori,S.R., and Kumamoto,C.A. (1999) Invasive lesions containing filamentous forms produced by a Candida albicans mutant that is defective in filamentous growth in culture. *Infect.Immun.* **67** (7), 3649-3652.

242. Sobel,J.D., Muller,G., and Buckley,H.R. (1984) Critical role of germ tube formation in the pathogenesis of candidal vaginitis. *Infect.Immun.* **44** (3), 576-580.

243. De Bernardis,F., Agatensi,L., Ross,I.K., Emerson,G.W., Lorenzini,R., Sullivan,P.A., and Cassone,A. (1990) Evidence for a role for secreted aspartate proteinase of Candida albicans in vulvovaginal candidiasis. *J.Infect.Dis.* **161** (6), 1276-1283.

244. Kelly,M.T., MacCallum,D.M., Clancy,S.D., Odds,F.C., Brown,A.J., and Butler,G. (2004) The Candida albicans CaACE2 gene affects morphogenesis, adherence and virulence. *Mol.Microbiol.* **53** (3), 969-983.

245. Torosantucci,A., Romagnoli,G., Chiani,P., Stringaro,A., Crateri,P., Mariotti,S., Teloni,R., Arancia,G., Cassone,A., and Nisini,R. (2004) Candida albicans yeast and germ tube forms interfere differently with human monocyte differentiation into dendritic cells: a novel dimorphism-dependent mechanism to escape the host's immune response. *Infect.Immun.* **72** (2), 833-843.

246. d'Ostiani,C.F., Del Sero,G., Bacci,A., Montagnoli,C., Spreca,A., Mencacci,A., Ricciardi-Castagnoli,P., and Romani,L. (2000) Dendritic cells discriminate between yeasts and hyphae of the fungus Candida albicans. Implications for initiation of T helper cell immunity in vitro and in vivo. *J Exp.Med.* **191** (10), 1661-1674.

247. Villar,C.C., Kashleva,H., and Dongari-Bagtzoglou,A. (2004) Role of Candida albicans polymorphism in interactions with oral epithelial cells. *Oral Microbiol.Immunol.* **19** (4), 262-269.

248. Saville,S.P., Lazzell,A.L., Monteagudo,C., and Lopez-Ribot,J.L. (2003) Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of Candida albicans during infection. *Eukaryot.Cell* **2** (5), 1053-1060.

249. Bendel,C.M., Hess,D.J., Garni,R.M., Henry-Stanley,M., and Wells,C.L. (2003) Comparative virulence of Candida albicans yeast and filamentous forms in orally and intravenously inoculated mice. *Crit Care Med.* **31** (2), 501-507.

250. Hogan,D.A. and Kolter,R. (2002) Pseudomonas-Candida interactions: an ecological role for virulence factors. *Science* **296** (5576), 2229-2232.

251. Hoshika,K. and Mine,H. (1994) Significance of modes of adherence in esophageal Candida albicans. *J.Gastroenterol.* **29** (1), 1-5.

252. Hoshika,K., Iida,M., and Mine,H. (1996) Esophageal Candida infection and adherence mechanisms in the nonimmunocompromised rabbit. *J.Gastroenterol.* **31** (3), 307-313.

253. Calderone,R.A. and Fonzi,W.A. (2001) Virulence factors of Candida albicans. *Trends Microbiol.* **9** (7), 327-335.

254. Kodsi,B.E., Wickremesinghe,C., Kozinn,P.J., Iswara,K., and Goldberg,P.K. (1976) Candida esophagitis: a prospective study of 27 cases. *Gastroenterology* **71** (5), 715-719.

255. Casanova,M., Gil,M.L., Cardenoso,L., Martinez,J.P., and Sentandreu,R. (1989) Identification of wall-specific antigens synthesized during germ tube formation by Candida albicans. *Infect.Immun.* **57** (1), 262-271.

256. Brawner,D.L. and Cutler,J.E. (1986) Variability in expression of cell surface antigens of Candida albicans during morphogenesis. *Infect.Immun.* **51** (1), 337-343.

257. Casanova,M., Martinez,J.P., and Chaffin,W.L. (1991) Identification of germ tube cell wall antigens of Candida albicans. *J.Med.Vet.Mycol.* **29** (4), 269-272.

258. Casanova,M., Lopez-Ribot,J.L., Martinez,J.P., and Sentandreu,R. (1992) Characterization of cell wall proteins from yeast and mycelial cells of Candida albicans by labelling with biotin: comparison with other techniques. *Infect.Immun.* **60** (11), 4898-4906.

259. Hazen,K.C. and Hazen,B.W. (1987) Temperature-modulated physiological characteristics of Candida albicans. *Microbiol.Immunol.* **31** (6), 497-508.

260. Hazen,B.W. and Hazen,K.C. (1988) Dynamic expression of cell surface hydrophobicity during initial yeast cell growth and before germ tube formation of Candida albicans. *Infect.Immun.* **56** (9), 2521-2525.

261. Lopez-Ribot,J.L., Casanova,M., Martinez,J.P., and Sentandreu,R. (1991) Characterization of cell wall proteins of yeast and hydrophobic mycelial cells of Candida albicans. *Infect.Immun.* **59** (7), 2324-2332.

262. Bouali,A., Robert,R., Tronchin,G., and Senet,J.M. (1987) Characterization of binding of human fibrinogen to the surface of germ-tubes and mycelium of candida albicans. *J.Gen.Microbiol.* **133** (3), 545-551.

263. Bouchara,J.P., Tronchin,G., Annaix,V., Robert,R., and Senet,J.M. (1990) Laminin receptors on Candida albicans germ tubes. *Infect.Immun.* **58** (1), 48-54.

264. Calderone,R.A. and Wadsworth,E. (1988) Characterization of mannoproteins from a virulent Candida albicans strain and its derived, avirulent strain. *Rev.Infect.Dis.* **10 Suppl 2** S423-S427.

265. Casanova,M., Lopez-Ribot,J.L., Monteagudo,C., Llombart-Bosch,A., Sentandreu,R., and Martinez,J.P. (1992) Identification of a 58-kilodalton cell surface fibrinogen-binding mannoprotein from Candida albicans. *Infect.Immun.* **60** (10), 4221-4229.

266. Slutsky,B., Buffo,J., and Soll,D.R. (1985) High-frequency switching of colony morphology in Candida albicans. *Science* **230** (4726), 666-669.

267. Vargas,K., Wertz,P.W., Drake,D., Morrow,B., and Soll,D.R. (1994) Differences in adhesion of Candida albicans 3153A cells exhibiting switch phenotypes to buccal epithelium and stratum corneum. *Infect.Immun.* **62** (4), 1328-1335.

268. Kvaal,C., Lachke,S.A., Srikantha,T., Daniels,K., McCoy,J., and Soll,D.R. (1999) Misexpression of the opaque-phase-specific gene PEP1 (SAP1) in the white phase of Candida albicans confers increased virulence in a mouse model of cutaneous infection. *Infect.Immun.* **67** (12), 6652-6662.

269. Soll,D.R. and Srikantha,T. (1998) Reporters for the analysis of gene regulation in fungi pathogenic to man. *Curr.Opin.Microbiol.* **1** (4), 400-405.

270. Kwon-Chung,K.J., Lehman,D., Good,C., and Magee,P.T. (1985) Genetic evidence for role of extracellular proteinase in virulence of Candida albicans. *Infect.Immun.* **49** (3), 571-575.

271. Cassone,A., De Bernardis,F., Mondello,F., Ceddia,T., and Agatensi,L. (1987) Evidence for a correlation between proteinase secretion and vulvovaginal candidosis. *J.Infect.Dis.* **156** (5), 777-783.

272. Naglik,J.R., Challacombe,S.J., and Hube,B. (2003) Candida albicans secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol.Mol.Biol.Rev.* **67** (3), 400-28, table.

273. Hube,B. (1996) Candida albicans secreted aspartyl proteinases. *Curr.Top.Med.Mycol.* **7** (1), 55-69.

274. Hube,B., Ruchel,R., Monod,M., Sanglard,D., and Odds,F.C. (1998) Functional aspects of secreted Candida proteinases. *Adv.Exp.Med.Biol.* **436** 339-344.

275. Morschhauser,J., Virkola,R., Korhonen,T.K., and Hacker,J. (1997) Degradation of human subendothelial extracellular matrix by proteinase-secreting Candida albicans. *FEMS Microbiol.Lett.* **153** (2), 349-355.

276. Colina,A.R., Aumont,F., Deslauriers,N., Belhumeur,P., and de Repentigny,L. (1996) Evidence for degradation of gastrointestinal mucin by Candida albicans secretory aspartyl proteinase. *Infect Immun.* **64** (11), 4514-4519.

277. Ruchel,R. (1986) Cleavage of immunoglobulins by pathogenic yeasts of the genus Candida. *Microbiol.Sci.* **3** (10), 316-319.

278. Kaminishi,H., Miyaguchi,H., Tamaki,T., Suenaga,N., Hisamatsu,M., Mihashi,I., Matsumoto,H., Maeda,H., and Hagihara,Y. (1995) Degradation of humoral host defense by Candida albicans proteinase. *Infect Immun.* **63** (3), 984-988.

279. Ruchel,R., Zimmermann,F., Boning-Stutzer,B., and Helmchen,U. (1991) Candidiasis visualised by proteinase-directed immunofluorescence. *Virchows Arch.A Pathol.Anat.Histopathol.* **419** (3), 199-202.

280. Hube,B., Sanglard,D., Schaller,M., Ibrahim,A., Odds,F.C., and Gow,N.A. (1998) [What functions do six different genes for secretory proteinases have in Candida albicans?]. *Mycoses* **41 Suppl 1** 47-50.

281. Schaller,M., Korting,H.C., Schafer,W., Bastert,J., Chen,W., and Hube,B. (1999) Secreted aspartic proteinase (Sap) activity contributes to tissue damage in a model of human oral candidosis. *Mol.Microbiol.* **34** (1), 169-180.

282. Naglik,J.R., Rodgers,C.A., Shirlaw,P.J., Dobbie,J.L., Fernandes-Naglik,L.L., Greenspan,D., Agabian,N., and Challacombe,S.J. (2003) Differential expression of Candida albicans secreted aspartyl proteinase and phospholipase B genes in humans correlates with active oral and vaginal infections. *J.Infect.Dis.* **188** (3), 469-479.

283. Schaller,M., Bein,M., Korting,H.C., Baur,S., Hamm,G., Monod,M., Beinhauer,S., and Hube,B. (2003) The secreted aspartyl proteinases Sap1 and Sap2 cause tissue damage in an in vitro model of vaginal candidiasis based on reconstituted human vaginal epithelium. *Infect.Immun.* **71** (6), 3227-3234.

284. De Bernardis,F., Arancia,S., Morelli,L., Hube,B., Sanglard,D., Schafer,W., and Cassone,A. (1999) Evidence that members of the secretory aspartyl proteinase gene family, in particular SAP2, are virulence factors for Candida vaginitis. *J.Infect.Dis.* **179** (1), 201-208.

285. Ibrahim,A.S., Mirbod,F., Filler,S.G., Banno,Y., Cole,G.T., Kitajima,Y., Edwards,J.E., Jr., Nozawa,Y., and Ghannoum,M.A. (1995) Evidence implicating phospholipase as a virulence factor of Candida albicans. *Infect Immun.* **63** (5), 1993-1998.

286. Mukherjee,P.K., Chandra,J., Kuhn,D.M., and Ghannoum,M.A. (2003) Differential expression of Candida albicans phospholipase B (PLB1) under various environmental and physiological conditions. *Microbiology* **149** (Pt 1), 261-267.

287. Leidich,S.D., Ibrahim,A.S., Fu,Y., Koul,A., Jessup,C., Vitullo,J., Fonzi,W., Mirbod,F., Nakashima,S., Nozawa,Y., and Ghannoum,M.A. (1998) Cloning and disruption of caPLB1, a phospholipase B gene involved in the pathogenicity of Candida albicans. *J Biol.Chem.* **273** (40), 26078-26086.

288. Mukherjee,P.K., Seshan,K.R., Leidich,S.D., Chandra,J., Cole,G.T., and Ghannoum,M.A. (2001) Reintroduction of the PLB1 gene into Candida albicans restores virulence in vivo. *Microbiology* **147** (Pt 9), 2585-2597.

289. Jayatilake,J.A., Samaranayake,Y.H., and Samaranayake,L.P. (2005) An ultrastructural and a cytochemical study of candidal invasion of reconstituted human oral epithelium. *J Oral Pathol.Med.* **34** (4), 240-246.

290. Persi,M.A., Burnham,J.C., and Duhring,J.L. (1985) Effects of carbon dioxide and pH on adhesion of Candida albicans to vaginal epithelial cells. *Infect.Immun.* **50** (1), 82-90.

291. Buffo,J., Herman,M.A., and Soll,D.R. (1984) A characterization of pH-regulated dimorphism in Candida albicans. *Mycopathologia* **85** (1-2), 21-30.

292. Fidel,P.L., Jr., Cutright,J., and Steele,C. (2000) Effects of reproductive hormones on experimental vaginal candidiasis. *Infect.Immun.* **68** (2), 651-657.

293. Steele,C., Ozenci,H., Luo,W., Scott,M., and Fidel,P.L., Jr. (1999) Growth inhibition of Candida albicans by vaginal cells from naive mice. *Med.Mycol.* **37** (4), 251-259.

294. Steele,C. and Fidel,P.L., Jr. (2002) Cytokine and chemokine production by human oral and vaginal epithelial cells in response to Candida albicans. *Infect Immun.* **70** (2), 577-583.

295. Hamad,M., Abu-Elteen,K.H., and Ghaleb,M. (2004) Estrogen-dependent induction of persistent vaginal candidosis in naive mice. *Mycoses* **47** (7), 304-309.

296. Dennerstein,G.J. and Ellis,D.H. (2001) Oestrogen, glycogen and vaginal candidiasis. *Aust.N.Z.J.Obstet.Gynaecol.* **41** (3), 326-328.

297. Kronenberg,M.S. and Clark,J.H. (1985) Changes in keratin expression during the estrogen-mediated differentiation of rat vaginal epithelium. *Endocrinology* **117** (4), 1480-1489.

298. Kronenberg,M.S. and Clark,J.H. (1985) Identification and analysis of keratin polypeptides from isolated rat vaginal epithelium. *Endocrinology* **117** (4), 1469-1479.

299. Gupta,P.D., Vijayasaradhi,S., and Reddy,A.G. (1989) Keratinization of rat vaginal epithelium. III. Effect of estradiol on keratinization. *Biol.Cell* **65** (3), 281-289.

300. Gimenez-Conti,I.B., Lynch,M., Roop,D., Bhowmik,S., Majeski,P., and Conti,C.J. (1994) Expression of keratins in mouse vaginal epithelium. *Differentiation* **56** (3), 143-151.

301. Williams,D.W., Walker,R., Lewis,M.A., Allison,R.T., and Potts,A.J. (1999) Adherence of Candida albicans to oral epithelial cells differentiated by Papanicolaou staining. *J.Clin.Pathol.* **52** (7), 529-531.

302. Hermann,P., Forgacs,K., Gal,E., Lenkey,B., Nagy,G., and Rozgonyi,F. (2003) Effects of alkali metal ions on some virulence traits of Candida albicans. *Folia Microbiol.(Praha)* **48** (2), 173-176.

303. Shepherd,M.G. (1987) Cell envelope of Candida albicans. *Crit Rev.Microbiol.* **15** (1), 7-25.

304. Pendrak,M.L. and Klotz,S.A. (1995) Adherence of Candida albicans to host cells. *FEMS Microbiol.Lett.* **129** (2-3), 103-113.

305. Yu,L., Lee,K.K., Sheth,H.B., Lane-Bell,P., Srivastava,G., Hindsgaul,O., Paranchych,W., Hodges,R.S., and Irvin,R.T. (1994) Fimbria-mediated adherence of Candida albicans to glycosphingolipid receptors on human buccal epithelial cells. *Infect.Immun.* **62** (7), 2843-2848.

306. Yu,L., Lee,K.K., Hodges,R.S., Paranchych,W., and Irvin,R.T. (1994) Adherence of Pseudomonas aeruginosa and Candida albicans to glycosphingolipid (Asialo-GM1) receptors is achieved by a conserved receptor-binding domain present on their adhesins. *Infect.Immun.* **62** (12), 5213-5219.

307. Hazen,K.C., Brawner,D.L., Riesselman,M.H., Jutila,M.A., and Cutler,J.E. (1991) Differential adherence of hydrophobic and hydrophilic Candida albicans yeast cells to mouse tissues. *Infect.Immun.* **59** (3), 907-912.

308. Macura,A.B. (1987) Hydrophobicity of Candida albicans related to their adherence to mucosal epithelial cells. *Zentralbl.Bakteriol.Mikrobiol.Hyg.[A]* **266** (3-4), 491-496.

309. Klotz,S.A., Drutz,D.J., and Zajic,J.E. (1985) Factors governing adherence of Candida species to plastic surfaces. *Infect Immun.* **50** (1), 97-101.

310. Goldmann,D.A. and Pier,G.B. (1993) Pathogenesis of infections related to intravascular catheterization. *Clinical Microbiology Reviews* **6** (2), 176-192.

311. Silva,T.M., Glee,P.M., and Hazen,K.C. (1995) Influence of cell surface hydrophobicity on attachment of Candida albicans to extracellular matrix proteins. *J.Med.Vet.Mycol.* **33** (2), 117-122.

312. Blanco,M.T., Blanco,J., Sanchez-Benito,R., Perez-Giraldo,C., Moran,F.J., Hurtado,C., and Gomez-Garcia,A.C. (1997) Incubation temperatures affect adherence to plastic of Candida albicans by changing the cellular surface hydrophobicity. *Microbios* **89** (358), 23-28.

313. Masuoka,J. and Hazen,K.C. (1997) Cell wall protein mannosylation determines Candida albicans cell surface hydrophobicity. *Microbiology* **143 (Pt 9)** 3015-3021.

314. Hazen,K.C. and Hazen,B.W. (1992) Hydrophobic surface protein masking by the opportunistic fungal pathogen Candida albicans. *Infect.Immun.* **60** (4), 1499-1508.

315. Li,X., Yan,Z., and Xu,J. (2003) Quantitative variation of biofilms among strains in natural populations of Candida albicans. *Microbiology* **149** (Pt 2), 353-362.

316. Cannon,R.D. and Chaffin,W.L. (1999) Oral colonization by Candida albicans. *Crit Rev.Oral Biol.Med.* **10** (3), 359-383.

317. Jabra-Rizk,M.A., Falkler,W.A., Jr., Merz,W.G., Baqui,A.A., Kelley,J.I., and Meiller,T.F. (2001) Cell surface hydrophobicity-associated adherence of Candida dubliniensis to human buccal epithelial cells. *Rev.Iberoam.Micol.* **18** (1), 17-22.

318. Antley,P.P. and Hazen,K.C. (1988) Role of yeast cell growth temperature on Candida albicans virulence in mice. *Infect.Immun.* **56** (11), 2884-2890.

319. Hazen,K.C., Mandell,G., Coleman,E., and Wu,G. (2000) Influence of fluconazole at subinhibitory concentrations on cell surface hydrophobicity and phagocytosis of Candida albicans. *FEMS Microbiol.Lett.* **183** (1), 89-94.

320. Peltroche-Llacsahuanga,H., Schnitzler,N., Schmidt,S., Tintelnot,K., Lutticken,R., and Haase,G. (2000) Phagocytosis, oxidative burst, and killing of Candida dubliniensis and Candida albicans by human neutrophils. *FEMS Microbiol Lett.* **191** (1), 151-155.

321. Kimura,L.H. and Pearsall,N.N. (1978) Adherence of Candida albicans to human buccal epithelial cells. *Infect.Immun.* **21** (1), 64-68.

322. Samaranayake,L.P. and MacFarlane,T.W. (1982) The effect of dietary carbohydrates on the in-vitro adhesion of Candida albicans to epithelial cells. *J.Med.Microbiol.* **15** (4), 511-517.

323. Fukazawa,Y. and Kagaya,K. (1997) Molecular bases of adhesion of Candida albicans. *J.Med.Vet.Mycol.* **35** (2), 87-99.

324. Hazen,K.C. and Hazen,B.W. (1993) Surface hydrophobic and hydrophilic protein alterations in Candida albicans. *FEMS Microbiol.Lett.* **107** (1), 83-87.

325. Klotz,S.A. and Maca,R.D. (1988) Endothelial cell contraction increases Candida adherence to exposed extracellular matrix. *Infect.Immun.* **56** (9), 2495-2498.

326. Klotz,S.A. and Smith,R.L. (1995) Gelatin fragments block adherence of Candida albicans to extracellular matrix proteins. *Microbiology* **141 (Pt 10)** 2681-2684.

327. Vercellotti,G.M., Platt,J.L., Bach,F.H., and Dalmasso,A.P. (1991) Neutrophil adhesion to xenogeneic endothelium via iC3b. *J.Immunol.* **146** (2), 730-734.

328. Edwards,J.E., Jr., Mayer,C.L., Filler,S.G., Wadsworth,E., and Calderone,R.A. (1992) Cell extracts of Candida albicans block adherence of the organisms to endothelial cells. *Infect.Immun.* **60** (8), 3087-3091.

329. Lee,K.H., Yoon,M.S., and Chun,W.H. (1997) The effects of monoclonal antibodies against iC3b receptors in mice with experimentally induced disseminated candidiasis. *Immunology* **92** (1), 104-110.

330. Wurzner,R., Langgartner,M., Spotl,L., Eder,A., Bujdakova,H., Schroppel,K., and Dierich,M.P. (1996) Temperature-dependent surface expression of the beta-2-integrin analogue of Candida albicans and its role in adhesion to the human endothelium. *Exp.Clin.Immunogenet.* **13** (3-4), 161-172.

331. Gustafson,K.S., Vercellotti,G.M., Bendel,C.M., and Hostetter,M.K. (1991) Molecular mimicry in Candida albicans. Role of an integrin analogue in adhesion of the yeast to human endothelium. *J.Clin.Invest* **87** (6), 1896-1902.

332. Goins,T.L. and Cutler,J.E. (2000) Relative abundance of oligosaccharides in Candida species as determined by fluorophore-assisted carbohydrate electrophoresis. *J Clin.Microbiol* **38** (8), 2862-2869.

333. Gil,M.L., Casanova,M., Martinez,J.P., and Sentandreu,R. (1991) Antigenic cell wall mannoproteins in Candida albicans isolates and in other Candida species. *J.Gen.Microbiol.* **137** (5), 1053-1061.

334. Martinez,J.P., Gil,M.L., Casanova,M., Lopez-Ribot,J.L., Garcia,D.L., and Sentandreu,R. (1990) Wall mannoproteins in cells from colonial phenotypic variants of Candida albicans. *J.Gen.Microbiol.* **136** (12), 2421-2432.

335. Calderone,R.A. (1993) Recognition between Candida albicans and host cells. *Trends Microbiol.* **1** (2), 55-58.

336. Reinhart,H., Muller,G., and Sobel,J.D. (1985) Specificity and mechanism of in vitro adherence of Candida albicans. *Ann.Clin.Lab Sci.* **15** (5), 406-413.

337. Segal,E., Lehrer,N., and Ofek,I. (1982) Adherence of Candida albicans to human vaginal epithelial cells: inhibition by amino sugars. *Exp.Cell Biol.* **50** (1), 13-17.

338. Segal,E., Gottfried,L., and Lehrer,N. (1988) Candidal vaginitis in hormone-treated mice: prevention by a chitin extract. *Mycopathologia* **102** (3), 157-163.

339. Lehrer,N., Segal,E., Lis,H., and Gov,Y. (1988) Effect of Candida albicans cell wall components on the adhesion of the fungus to human and murine vaginal mucosa. *Mycopathologia* **102** (2), 115-121.

340. Colling,L., Carter,R.N., Essmann,M., and Larsen,B. (2005) Evaluation of relative yeast cell surface hydrophobicity measured by flow cytometry. *Infect.Dis.Obstet.Gynecol.* **13** (1), 43-48.

341. Segal,E., Kremer,I., and Dayan,D. (1992) Inhibition of adherence of Candida albicans to acrylic by a chitin derivative. *Eur.J.Epidemiol.* **8** (3), 350-355.

342. Santoni,G., Birarelli,P., Hong,L.J., Gamero,A., Djeu,J.Y., and Piccoli,M. (1995) An alpha 5 beta 1-like integrin receptor mediates the binding of less pathogenic Candida species to fibronectin. *J.Med.Microbiol.* **43** (5), 360-367.

343. Lopez-Ribot,J.L., Monteagudo,C., Sepulveda,P., Casanova,M., Martinez,J.P., and Chaffin,W.L. (1996) Expression of the fibrinogen binding mannoprotein and the laminin receptor of Candida albicans in vitro and in infected tissues. *FEMS Microbiol.Lett.* **142** (1), 117-122.

344. Ollert,M.W., Sohnchen,R., Korting,H.C., Ollert,U., Brautigam,S., and Brautigam,W. (1993) Mechanisms of adherence of Candida albicans to cultured human epidermal keratinocytes. *Infect.Immun.* **61** (11), 4560-4568.

345. Santoni,G., Spreghini,E., Lucciarini,R., Amantini,C., and Piccoli,M. (2001) Involvement of alpha(v)beta3 integrin-like receptor and glycosaminoglycans in Candida albicans germ tube adhesion to vitronectin and to a human endothelial cell line. *Microb.Pathog.* **31** (4), 159-172.

346. Spreghini,E., Gismondi,A., Piccoli,M., and Santoni,G. (1999) Evidence for alphavbeta3 and alphavbeta5 integrin-like vitronectin (VN) receptors in Candida albicans and their involvement in yeast cell adhesion to VN. *J.Infect.Dis.* **180** (1), 156-166.

347. Makihira,S., Nikawa,H., Tamagami,M., Hamada,T., and Samaranayake,L.P. (2002) Differences in Candida albicans adhesion to intact and denatured type I collagen in vitro. *Oral Microbiol.Immunol.* **17** (2), 129-131.

348. Lopez-Ribot,J.L., Martinez,J.P., and Chaffin,W.L. (1995) Comparative study of the C3d receptor and 58-kilodalton fibrinogen-binding mannoproteins of Candida albicans. *Infect Immun.* **63** (6), 2126-2132.

349. Bouali,A., Robert,R., Tronchin,G., and Senet,J.M. (1986) Binding of human fibrinogen to Candida albicans in vitro: a preliminary study. *J Med.Vet.Mycol.* **24** (4), 345-348.

350. Tronchin,G., Robert,R., Bouali,A., and Senet,J.M. (1987) Immunocytochemical localization of in vitro binding of human fibrinogen to Candida albicans germ tube and mycelium. *Ann.Inst.Pasteur Microbiol* **138** (2), 177-187.

351. Tronchin,G., Bouchara,J.P., and Robert,R. (1989) Dynamic changes of the cell wall surface of Candida albicans associated with germination and adherence. *Eur.J Cell Biol.* **50** (2), 285-290.

352. Glee,P.M., Sundstrom,P., and Hazen,K.C. (1995) Expression of surface hydrophobic proteins by Candida albicans in vivo. *Infect.Immun.* **63** (4), 1373-1379.

353. Glee,P.M., Masuoka,J., Ozier,W.T., and Hazen,K.C. (1996) Presence of multiple laminin- and fibronectin-binding proteins in cell wall extract of Candida albicans: influence of dialysis. *J.Med.Vet.Mycol.* **34** (1), 57-61.

354. Alonso,R., Llopis,I., Flores,C., Murgui,A., and Timoneda,J. (2001) Different adhesins for type IV collagen on Candida albicans: identification of a lectin-like adhesin recognizing the 7S(IV) domain. *Microbiology* **147** (Pt 7), 1971-1981.

355. Cotter,G., Weedle,R., and Kavanagh,K. (1998) Monoclonal antibodies directed against extracellular matrix proteins reduce the adherence of Candida albicans to HEp-2 cells. *Mycopathologia* **141** (3), 137-142.

356. Gozalbo,D., Gil-Navarro,I., Azorin,I., Renau-Piqueras,J., Martinez,J.P., and Gil,M.L. (1998) The cell wall-associated glyceraldehyde-3-phosphate dehydrogenase of Candida albicans is also a fibronectin and laminin binding protein. *Infect.Immun.* **66** (5), 2052-2059.

357. Gil,M.L., Villamon,E., Monteagudo,C., Gozalbo,D., and Martinez,J.P. (1999) Clinical strains of Candida albicans express the surface antigen glyceraldehyde 3-phosphate dehydrogenase in vitro and in infected tissues. *FEMS Immunol.Med.Microbiol.* **23** (3), 229-234.

358. Lopez-Ribot,J.L., Bikandi,J., San Millan,R.S., and Chaffin,W.L. (1999) Interactions between Candida albicans and the human extracellular matrix component tenascin-C. *Mol.Cell Biol.Res.Commun.* **2** (1), 58-63.

359. Klotz,S.A., Smith,R.L., and Stewart,B.W. (1992) Effect of an arginine-glycine-aspartic acid-containing peptide on hematogenous candidal infections in rabbits. *Antimicrob.Agents Chemother.* **36** (1), 132-136.

360. Klotz,S.A., Gaur,N.K., Rauceo,J., Lake,D.F., Park,Y., Hahm,K.S., and Lipke,P.N. (2004) Inhibition of adherence and killing of Candida albicans with a 23-Mer peptide (Fn/23) with dual antifungal properties. *Antimicrob.Agents Chemother.* **48** (11), 4337-4341.

361. Calderone,R.A., Linehan,L., Wadsworth,E., and Sandberg,A.L. (1988) Identification of C3d receptors on Candida albicans. *Infect.Immun.* **56** (1), 252-258.

362. Wadsworth,E., Prasad,S.C., and Calderone,R. (1993) Analysis of mannoproteins from blastoconidia and hyphae of Candida albicans with a common epitope recognized by anti-complement receptor type 2 antibodies. *Infect.Immun.* **61** (11), 4675-4681.

363. Hostetter,M.K., Lorenz,J.S., Preus,L., and Kendrick,K.E. (1990) The iC3b receptor on Candida albicans: subcellular localization and modulation of receptor expression by glucose. *J.Infect.Dis.* **161** (4), 761-768.

364. Miyakawa,Y., Kagaya,K., Kuribayashi,T., Suzuki,M., and Fukazawa,Y. (1989) Isolation and chemical and biological characterization of antigenic mutants of Candida albicans serotype A. *Yeast* **5 Spec No** S225-S229.

365. Ghannoum,M.A., Abu el-Teen,K., and Radwan,S.S. (1987) Blocking adherence of Candida albicans to buccal epithelial cells by yeast glycolipids, yeast wall lipids and lipids from epithelial cells. *Mykosen* **30** (8), 371-378.

366. Maisch,P.A. and Calderone,R.A. (1981) Role of surface mannan in the adherence of Candida albicans to fibrin-platelet clots formed in vitro. *Infect.Immun.* **32** (1), 92-97.

367. Sandin,R.L. and Rogers,A.L. (1982) Inhibition of adherence of Candida albicans to human epithelial cells. *Mycopathologia* **77** (1), 23-26.

368. Sandin,R.L., Rogers,A.L., Patterson,R.J., and Beneke,E.S. (1982) Evidence for mannose-mediated adherence of Candida albicans to human buccal cells in vitro. *Infect.Immun.* **35** (1), 79-85.

369. Munro,C.A., Bates,S., Buurman,E.T., Hughes,H.B., MacCallum,D.M., Bertram,G., Atrih,A., Ferguson,M.A., Bain,J.M., Brand,A., Hamilton,S., Westwater,C., Thomson,L.M., Brown,A.J., Odds,F.C., and Gow,N.A. (2005) Mnt1p and Mnt2p of Candida albicans are partially redundant alpha-1,2-mannosyltransferases that participate in O-linked mannosylation and are required for adhesion and virulence. *J.Biol.Chem.* **280** (2), 1051-1060.

370. Hayette,M.P., Strecker,G., Faille,C., Dive,D., Camus,D., Mackenzie,D.W., and Poulain,D. (1992) Presence of human antibodies reacting with Candida albicans O-linked oligomannosides revealed by using an enzyme-linked immunosorbent assay and neoglycolipids. *J Clin.Microbiol* **30** (2), 411-417.

371. Lee,J.C. and King,R.D. (1983) Characterization of Candida albicans adherence to human vaginal epithelial cells in vitro. *Infect.Immun.* **41** (3), 1024-1030.

372. Centeno,A., Davis,C.P., Cohen,M.S., and Warren,M.M. (1983) Modulation of Candida albicans attachment to human epithelial cells by bacteria and carbohydrates. *Infect.Immun.* **39** (3), 1354-1360.

373. Tosh,F.D. and Douglas,L.J. (1992) Characterization of a fucoside-binding adhesin of Candida albicans. *Infect.Immun.* **60** (11), 4734-4739.

374. Cameron,B.J. and Douglas,L.J. (1996) Blood group glycolipids as epithelial cell receptors for Candida albicans. *Infect.Immun.* **64** (3), 891-896.

375. Lee,K.K., Yu,L., Macdonald,D.L., Paranchych,W., Hodges,R.S., and Irvin,R.T. (1996) Anti-adhesin antibodies that recognize a receptor-binding motif (adhesintope) inhibit pilus/fimbrial-mediated adherence of Pseudomonas aeruginosa and Candida albicans to asialo-GM1 receptors and human buccal epithelial cell surface receptors. *Can.J.Microbiol.* **42** (5), 479-486.

376. Brassart,D., Woltz,A., Golliard,M., and Neeser,J.R. (1991) In vitro inhibition of adhesion of Candida albicans clinical isolates to human buccal epithelial cells by Fuc alpha 1----2Gal beta-bearing complex carbohydrates. *Infect.Immun.* **59** (5), 1605-1613.

377. Staib,P., Kretschmar,M., Nichterlein,T., Kohler,G., Michel,S., Hof,H., Hacker,J., and Morschhauser,J. (1999) Host-induced, stage-specific virulence gene activation in Candida albicans during infection. *Mol.Microbiol.* **32** (3), 533-546.

378. Ghannoum,M.A., Abu-Elteen,K., Ibrahim,A., and Stretton,R. (1991) Protection against Candida albicans gastrointestinal colonization and dissemination by saccharides in experimental animals. *Microbios* **67** (271), 95-105.

379. Segal,E. and Savage,D.C. (1986) Adhesion of Candida albicans to mouse intestinal mucosa in vitro: development of the assay and test of inhibitors. *J.Med.Vet.Mycol.* **24** (6), 477-479.

380. Watts,H.J., Cheah,F.S., Hube,B., Sanglard,D., and Gow,N.A. (1998) Altered adherence in strains of Candida albicans harbouring null mutations in secreted aspartic proteinase genes. *FEMS Microbiol.Lett.* **159** (1), 129-135.

381. Schaller,M., Korting,H.C., Borelli,C., Hamm,G., and Hube,B. (2005) Candida albicans-secreted aspartic proteinases modify the epithelial cytokine response in an in vitro model of vaginal candidiasis. *Infect.Immun.* **73** (5), 2758-2765.

382. Albrecht,A., Felk,A., Pichova,I., Naglik,J.R., Schaller,M., de Groot,P., Maccallum,D., Odds,F.C., Schafer,W., Klis,F., Monod,M., and Hube,B. (2005) Glycosylphosphatidylinositol-anchored proteases of Candida albicans target proteins necessary for both cellular processes and host-pathogen interactions. *J.Biol.Chem.*

383. Omaetxebarria,M.J., Moragues,M.D., Elguezabal,N., Rodriguez-Alejandre,A., Brena,S., Schneider,J., Polonelli,L., and Ponton,J. (2005) Antifungal and antitumor activities of a monoclonal antibody directed against a stress mannoprotein of Candida albicans. *Curr.Mol.Med.* **5** (4), 393-401.

384. Swoboda,R.K., Bertram,G., Hollander,H., Greenspan,D., Greenspan,J.S., Gow,N.A., Gooday,G.W., and Brown,A.J. (1993) Glycolytic enzymes of Candida albicans are nonubiquitous immunogens during candidiasis. *Infect.Immun.* **61** (10), 4263-4271.

385. Mitsutake,K., Kohno,S., Miyazaki,T., Miyazaki,H., Maesaki,S., and Koga,H. (1994) Detection of Candida enolase antibody in patients with candidiasis. *J.Clin.Lab Anal.* **8** (4), 207-210.

386. Breitenbach,M., Simon,B., Probst,G., Oberkofler,H., Ferreira,F., Briza,P., Achatz,G., Unger,A., Ebner,C., Kraft,D., and Hirschwehr,R. (1997) Enolases are highly conserved fungal allergens. *Int.Arch.Allergy Immunol.* **113** (1-3), 114-117.

387. Singleton,D.R., Masuoka,J., and Hazen,K.C. (2001) Cloning and analysis of a Candida albicans gene that affects cell surface hydrophobicity. *J.Bacteriol.* **183** (12), 3582-3588.

388. Singleton,D.R., Fidel,P.L., Jr., Wozniak,K.L., and Hazen,K.C. (2005) Contribution of cell surface hydrophobicity protein 1 (Csh1p) to virulence of hydrophobic Candida albicans serotype A cells. *FEMS Microbiol.Lett.* **244** (2), 373-377.

389. Klotz,S.A., Pendrak,M.L., and Hein,R.C. (2001) Antibodies to alpha5beta1 and alpha(v)beta3 integrins react with Candida albicans alcohol dehydrogenase. *Microbiology* **147** (Pt 11), 3159-3164.

390. Alberti-Segui,C., Morales,A.J., Xing,H., Kessler,M.M., Willins,D.A., Weinstock,K.G., Cottarel,G., Fechtel,K., and Rogers,B. (2004) Identification of potential cell-surface proteins in Candida albicans and investigation of the role of a putative cell-surface glycosidase in adhesion and virulence. *Yeast* **21** (4), 285-302.

391. Zhao,X., Oh,S.H., Cheng,G., Green,C.B., Nuessen,J.A., Yeater,K., Leng,R.P., Brown,A.J., and Hoyer,L.L. (2004) ALS3 and ALS8 represent a single locus that encodes a Candida albicans adhesin; functional comparisons between Als3p and Als1p. *Microbiology* **150** (Pt 7), 2415-2428.

392. Loza,L., Fu,Y., Ibrahim,A.S., Sheppard,D.C., Filler,S.G., and Edwards,J.E., Jr. (2004) Functional analysis of the Candida albicans ALS1 gene product. *Yeast* **21** (6), 473-482.

393. Klotz,S.A., Gaur,N.K., Lake,D.F., Chan,V., Rauceo,J., and Lipke,P.N. (2004) Degenerate peptide recognition by Candida albicans adhesins Als5p and Als1p. *Infect.Immun.* **72** (4), 2029-2034.

394. Fu,Y., Ibrahim,A.S., Sheppard,D.C., Chen,Y.C., French,S.W., Cutler,J.E., Filler,S.G., and Edwards,J.E., Jr. (2002) Candida albicans Als1p: an adhesin that is a downstream effector of the EFG1 filamentation pathway. *Mol.Microbiol.* **44** (1), 61-72.

395. Zhao,X., Oh,S.H., Yeater,K.M., and Hoyer,L.L. (2005) Analysis of the Candida albicans Als2p and Als4p adhesins suggests the potential for compensatory function within the Als family. *Microbiology* **151** (Pt 5), 1619-1630.

396. Gaur,N.K., Smith,R.L., and Klotz,S.A. (2002) Candida albicans and Saccharomyces cerevisiae expressing ALA1/ALS5 adhere to accessible threonine, serine, or alanine patches. *Cell Commun.Adhes.* **9** (1), 45-57.

397. Gaur,N.K. and Klotz,S.A. (2004) Accessibility of the peptide backbone of protein ligands is a key specificity determinant in Candida albicans SRS adherence. *Microbiology* **150** (Pt 2), 277-284.

398. Ghannoum,M. and Abu,E.K. (1986) Correlative relationship between proteinase production, adherence and pathogenicity of various strains of Candida albicans. *J.Med.Vet.Mycol.* **24** (5), 407-413.

399. Li,F. and Palecek,S.P. (2003) EAP1, a Candida albicans gene involved in binding human epithelial cells. *Eukaryot.Cell* **2** (6), 1266-1273.
400. Bain,J.M., Stubberfield,C., and Gow,N.A. (2001) Ura-status-dependent adhesion of Candida albicans mutants. *FEMS Microbiol.Lett.* **204** (2), 323-328.

401. Cheng,S., Clancy,C.J., Checkley,M.A., Handfield,M., Hillman,J.D., Progulske-Fox,A., Lewin,A.S., Fidel,P.L., and Nguyen,M.H. (2003) Identification of Candida albicans genes induced during thrush offers insight into pathogenesis. *Mol.Microbiol.* **48** (5), 1275-1288.

402. Cheng,S., Nguyen,M.H., Zhang,Z., Jia,H., Handfield,M., and Clancy,C.J. (2003) Evaluation of the roles of four Candida albicans genes in virulence by using gene disruption strains that express URA3 from the native locus. *Infect Immun.* **71** (10), 6101-6103.

403. Naidu,A.S., Chen,J., Martinez,C., Tulpinski,J., Pal,B.K., and Fowler,R.S. (2004) Activated lactoferrin's ability to inhibit Candida growth and block yeast adhesion to the vaginal epithelial monolayer. *J.Reprod.Med.* **49** (11), 859-866.

404. Jimenez-Lucho,V., Ginsburg,V., and Krivan,H.C. (1990) Cryptococcus neoformans, Candida albicans, and other fungi bind specifically to the glycosphingolipid lactosylceramide (Gal beta 1-4Glc beta 1-1Cer), a possible adhesion receptor for yeasts. *Infect.Immun.* **58** (7), 2085-2090.

405. Nikawa,H. and Hamada,T. (1990) Binding of salivary or serum proteins to Candida albicans in vitro. *Arch.Oral Biol.* **35** (7), 571-573.

406. Hoffman,M.P. and Haidaris,C.G. (1993) Analysis of Candida albicans adhesion to salivary mucin. *Infect.Immun.* **61** (5), 1940-1949.

407. Hoffman,M.P. and Haidaris,C.G. (1994) Identification and characterization of a Candida albicans-binding proteoglycan secreted from rat submandibular salivary glands. *Infect.Immun.* **62** (3), 828-836.

408. de Repentigny,L., Aumont,F., Bernard,K., and Belhumeur,P. (2000) Characterization of binding of Candida albicans to small intestinal mucin and its role in adherence to mucosal epithelial cells. *Infect.Immun.* **68** (6), 3172-3179.

409. O'Sullivan,J.M., Cannon,R.D., Sullivan,P.A., and Jenkinson,H.F. (1997) Identification of salivary basic proline-rich proteins as receptors for Candida albicans adhesion. *Microbiology* **143 (Pt 2)** 341-348.

410. Powell,B.L. and Drutz,D.J. (1983) Confirmation of corticosterone and progesterone binding activity in Candida albicans. *J Infect Dis* **147** (2), 359.

411. Vardar-Unlu,G., McSharry,C., and Douglas,L.J. (1998) Fucose-specific adhesins on germ tubes of Candida albicans. *FEMS Immunol.Med.Microbiol.* **20** (1), 55-67.

412. Straube,E., Schmidt,G., Marre,R., and Hacker,J. (1993) Adhesion and internalization of E. coli strains expressing various pathogenicity determinants. *Zentralbl.Bakteriol.* **278** (2-3), 218-228.

413. Drago,L., Mombelli,B., De Vecchi,E., Bonaccorso,C., Fassina,M.C., and Gismondo,M.R. (2000) Candida albicans cellular internalization: a new pathogenic factor? *Int.J.Antimicrob.Agents* **16** (4), 545-547.

414. Bassilana,M., Blyth,J., and Arkowitz,R.A. (2003) Cdc24, the GDP-GTP exchange factor for Cdc42, is required for invasive hyphal growth of Candida albicans. *Eukaryot.Cell* **2** (1), 9-18.

415. Calera,J.A. and Calderone,R. (1999) Histidine kinase, two-component signal transduction proteins of Candida albicans and the pathogenesis of candidosis. *Mycoses* **42 Suppl 2** 49-53.

416. Sherwood,J., Gow,N.A., Gooday,G.W., Gregory,D.W., and Marshall,D. (1992) Contact sensing in Candida albicans: a possible aid to epithelial penetration. *J.Med.Vet.Mycol.* **30** (6), 461-469.

417. Fidel,P.L., Jr. (2002) Immunity to Candida. *Oral Dis* **8 Suppl 2** 69-75.

418. Vespa,M.N., Lopez-Ribot,J.L., and Chaffin,W.L. (1995) Adherence of germ tubes of Candida albicans to tissues from immunocompromised mice. *FEMS Immunol.Med.Microbiol.* **11** (1), 57-63.

419. Kullberg,B.J., Netea,M.G., Vonk,A.G., and van der Meer,J.W. (1999) Modulation of neutrophil function in host defense against disseminated Candida albicans infection in mice. *FEMS Immunol.Med.Microbiol* **26** (3-4), 299-307.

420. Van 't Wout,J.W., Linde,I., Leijh,P.C.J., and Van Furth,R. (1988) Contribution of granulocytes and monocytes to resistance against experimental disseminated *Candida albicans* infections. *Eur.J.Clin.Microbiol.Infect.Dis.* **7** 736-741.

421. Netea,M.G., Gijzen,K., Coolen,N., Verschueren,I., Figdor,C., van der Meer,J.W., Torensma,R., and Kullberg,B.J. (2004) Human dendritic cells are less potent at killing Candida albicans than both monocytes and macrophages. *Microbes.Infect.* **6** (11), 985-989.

422. Rodier,M.H., Imbert,C., Kauffmann-Lacroix,C., Daniault,G., and Jacquemin,J.L. (2003) Immunoglobulins G could prevent adherence of Candida albicans to polystyrene and extracellular matrix components. *J.Med.Microbiol.* **52** (Pt 5), 373-377.

423. Elguezabal,N., Maza,J.L., and Ponton,J. (2004) Inhibition of adherence of Candida albicans and Candida dubliniensis to a resin composite restorative dental material by salivary secretory IgA and monoclonal antibodies. *Oral Dis.* **10** (2), 81-86.

424. Reinholdt,J., Krogh,P., and Holmstrup,P. (1987) Degradation of IgA1, IgA2, and S-IgA by Candida and Torulopsis species. *Acta Pathol.Microbiol Immunol.Scand.[C.]* **95** (6), 265-274.

425. Monod,M. and Borg,v. (2002) Secreted aspartic proteases as virulence factors of Candida species. *Biol.Chem.* **383** (7-8), 1087-1093.

426. Feng,Z., Jiang,B., Chandra,J., Ghannoum,M., Nelson,S., and Weinberg,A. (2005) Human beta-defensins: differential activity against candidal species and regulation by Candida albicans. *J.Dent.Res.* **84** (5), 445-450.

427. Kozel,T.R., Weinhold,L.C., and Lupan,D.M. (1996) Distinct characteristics of initiation of the classical and alternative complement pathways by Candida albicans. *Infect Immun.* **64** (8), 3360-3368.

428. Ip,W.K. and Lau,Y.L. (2004) Role of mannose-binding lectin in the innate defense against Candida albicans: enhancement of complement activation, but lack of opsonic function, in phagocytosis by human dendritic cells. *J Infect Dis* **190** (3), 632-640.

429. Triebel,T., Grillhosl,B., Kacani,L., Lell,C.P., Fuchs,A., Speth,C., Lass-Florl,C., Steinmann,J., Dierich,M.P., and Wurzner,R. (2003) Importance of the terminal complement components for immune defence against Candida. *Int.J.Med.Microbiol.* **292** (7-8), 527-536.

430. Fidel,P.L., Jr. (2005) Immunity in vaginal candidiasis. *Curr.Opin.Infect Dis* **18** (2), 107-111.

431. Black,C.A., Eyers,F.M., Russell,A., Dunkley,M.L., Clancy,R.L., and Beagley,K.W. (1998) Acute neutropenia decreases inflammation associated with murine vaginal candidiasis but has no effect on the course of infection. *Infect Immun.* **66** (3), 1273-1275.

432. Fidel,P.L., Jr., Luo,W., Steele,C., Chabain,J., Baker,M., and Wormley,F., Jr. (1999) Analysis of vaginal cell populations during experimental vaginal candidiasis. *Infect Immun.* **67** (6), 3135-3140.

433. Fidel,P.L., Jr. and Sobel,J.D. (1996) Immunopathogenesis of recurrent vulvovaginal candidiasis. *Clinical Microbiology Reviews* **9** (3), 335-348.

434. Romani,L., Puccetti,P., and Bistoni,F. (1996) Biological role of Th cell subsets in candidiasis. *Chem.Immunol.* **63** 115-137.

435. Taylor,B.N., Saavedra,M., and Fidel,P.L., Jr. (2000) Local Th1/Th2 cytokine production during experimental vaginal candidiasis: potential importance of transforming growth factor-beta. *Med.Mycol.* **38** (6), 419-431.

436. Wormley,F.L., Jr., Steele,C., Wozniak,K., Fujihashi,K., McGhee,J.R., and Fidel,P.L., Jr. (2001) Resistance of T-cell receptor delta-chain-deficient mice to experimental Candida albicans vaginitis. *Infect Immun.* **69** (11), 7162-7164.

437. Romani,L., Mencacci,A., Cenci,E., Puccetti,P., and Bistoni,F. (1996) Neutrophils and the adaptive immune response to Candida albicans. *Res.Immunol.* **147** (8-9), 512-518.

438. Parsonnet,J. (1995) The incidence of Helicobacter pylori infection. *Aliment.Pharmacol.Ther.* **9 Suppl 2** 45-51.

439. Frenck,R.W., Jr. and Clemens,J. (2003) Helicobacter in the developing world. *Microbes.Infect.* **5** (8), 705-713.

440. Rothenbacher,D. and Brenner,H. (2003) Burden of Helicobacter pylori and H. pylori-related diseases in developed countries: recent developments and future implications. *Microbes.Infect.* **5** (8), 693-703.

441. Kikuchi,S. and Dore,M.P. (2005) Epidemiology of Helicobacter pylori Infection. *Helicobacter.* **10 Suppl 1** 1-4.

442. Czinn,S.J. (2005) Helicobacter pylori infection: detection, investigation, and management. *J Pediatr.* **146** (3 Suppl), S21-S26.

443. Podzorski,R.P., Podzorski,D.S., Wuerth,A., and Tolia,V. (2003) Analysis of the vacA, cagA, cagE, iceA, and babA2 genes in Helicobacter pylori from sixty-one pediatric patients from the Midwestern United States. *Diagn.Microbiol.Infect.Dis.* **46** (2), 83-88.

444. Montecucco,C. and Rappuoli,R. (2001) Living dangerously: how Helicobacter pylori survives in the human stomach. *Nat.Rev.Mol.Cell Biol.* **2** (6), 457-466.

445. Achtman, M. *Helicobacter pylori:* Molecular and Cellular Biology. Suerbaum, S. Norfolk, Horizon Scientific.
Ref Type: Generic

446. Scott,D.R., Weeks,D., Hong,C., Postius,S., Melchers,K., and Sachs,G. (1998) The role of internal urease in acid resistance of Helicobacter pylori. *Gastroenterology* **114** (1), 58-70.

447. Weeks,D.L., Eskandari,S., Scott,D.R., and Sachs,G. (2000) A H+-gated urea channel: the link between Helicobacter pylori urease and gastric colonization. *Science* **287** (5452), 482-485.

448. Dunn,B.E., Vakil,N.B., Schneider,B.G., Miller,M.M., Zitzer,J.B., Peutz,T., and Phadnis,S.H. (1997) Localization of Helicobacter pylori urease and heat shock protein in human gastric biopsies. *Infect.Immun.* **65** (4), 1181-1188.

449. Ha,N.C., Oh,S.T., Sung,J.Y., Cha,K.A., Lee,M.H., and Oh,B.H. (2001) Supramolecular assembly and acid resistance of Helicobacter pylori urease. *Nat.Struct.Biol.* **8** (6), 505-509.

450. Eaton,K.A., Brooks,C.L., Morgan,D.R., and Krakowka,S. (1991) Essential role of urease in pathogenesis of gastritis induced by Helicobacter pylori in gnotobiotic piglets. *Infect.Immun.* **59** (7), 2470-2475.

451. Clyne,M., Labigne,A., and Drumm,B. (1995) Helicobacter pylori requires an acidic environment to survive in the presence of urea. *Infect.Immun.* **63** (5), 1669-1673.

452. Williams,C.L., Preston,T., Hossack,M., Slater,C., and McColl,K.E. (1996) Helicobacter pylori utilises urea for amino acid synthesis. *FEMS Immunol.Med.Microbiol.* **13** (1), 87-94.

453. Megraud,F., Neman-Simha,V., and Brugmann,D. (1992) Further evidence of the toxic effect of ammonia produced by Helicobacter pylori urease on human epithelial cells. *Infect Immun.* **60** (5), 1858-1863.

454. Suzuki,M., Miura,S., Suematsu,M., Fukumura,D., Kurose,I., Suzuki,H., Kai,A., Kudoh,Y., Ohashi,M., and Tsuchiya,M. (1992) Helicobacter pylori-associated ammonia production enhances neutrophil-dependent gastric mucosal cell injury. *Am.J Physiol* **263** (5 Pt 1), G719-G725.

455. Sommi,P., Ricci,V., Fiocca,R., Romano,M., Ivey,K.J., Cova,E., Solcia,E., and Ventura,U. (1996) Significance of ammonia in the genesis of gastric epithelial lesions induced by Helicobacter pylori: an in vitro study with different bacterial strains and urea concentrations. *Digestion* **57** (5), 299-304.

456. Murakami,M., Yoo,J.K., Teramura,S., Yamamoto,K., Saita,H., Matuo,K., Asada,T., and Kita,T. (1990) Generation of ammonia and mucosal lesion formation following hydrolysis of urea by urease in the rat stomach. *J.Clin.Gastroenterol.* **12 Suppl 1** S104-S109.

457. Murakami,M., Saita,H., Teramura,S., Dekigai,H., Asagoe,K., Kusaka,S., and Kita,T. (1993) Gastric ammonia has a potent ulcerogenic action on the rat stomach. *Gastroenterology* **105** (6), 1710-1715.

458. Josenhans,C. and Suerbaum,S. (2002) The role of motility as a virulence factor in bacteria. *Int.J.Med.Microbiol.* **291** (8), 605-614.

459. Ottlecz,A., Romero,J.J., and Lichtenberger,L.M. (1999) Effect of ranitidine bismuth citrate on the phospholipase A2 activity of Naja naja venom and Helicobacter pylori: a biochemical analysis. *Aliment.Pharmacol.Ther.* **13** (7), 875-881.

460. Weitkamp,J.H., Perez-Perez,G.I., Bode,G., Malfertheiner,P., and Blaser,M.J. (1993) Identification and characterization of Helicobacter pylori phospholipase C activity. *Zentralbl.Bakteriol.* **280** (1-2), 11-27.

461. Pomorski,T., Meyer,T.F., and Naumann,M. (2001) Helicobacter pylori-induced prostaglandin E(2) synthesis involves activation of cytosolic phospholipase A(2) in epithelial cells. *J Biol.Chem.* **276** (1), 804-810.

462. Romani,L. and Kaufmann,S.H. (1998) Immunity to fungi: editorial overview. *Res.Immunol.* **149** (4-5), 277-281.

463. Noverr,M.C., Toews,G.B., and Huffnagle,G.B. (2002) Production of prostaglandins and leukotrienes by pathogenic fungi. *Infect Immun.* **70** (1), 400-402.

464. Censini,S., Lange,C., Xiang,Z., Crabtree,J.E., Ghiara,P., Borodovsky,M., Rappuoli,R., and Covacci,A. (1996) cag, a pathogenicity island of Helicobacter pylori, encodes type I-specific and disease-associated virulence factors. *Proc.Natl.Acad.Sci.U.S.A* **93** (25), 14648-14653.

465. Tanaka,J., Suzuki,T., Mimuro,H., and Sasakawa,C. (2003) Structural definition on the surface of Helicobacter pylori type IV secretion apparatus. *Cell Microbiol* **5** (6), 395-404.

466. Abe,A., Matsuzawa,T., and Kuwae,A. (2005) Type-III effectors: sophisticated bacterial virulence factors. *C.R.Biol.* **328** (5), 413-428.

467. Crabtree,J.E. (1996) Gastric mucosal inflammatory responses to Helicobacter pylori. *Aliment.Pharmacol.Ther.* **10 Suppl 1** 29-37.

468. Yamazaki,S., Yamakawa,A., Ito,Y., Ohtani,M., Higashi,H., Hatakeyama,M., and Azuma,T. (2003) The CagA protein of Helicobacter pylori is translocated into epithelial cells and binds to SHP-2 in human gastric mucosa. *J.Infect.Dis.* **187** (2), 334-337.
469. Parsonnet,J., Replogle,M., Yang,S., and Hiatt,R. (1997) Seroprevalence of CagA-positive strains among Helicobacter pylori-infected, healthy young adults. *J.Infect.Dis.* **175** (5), 1240-1242.

470. Atherton,J.C. (1998) H. pylori virulence factors. *Br.Med.Bull.* **54** (1), 105-120.

471. Tummuru,M.K., Cover,T.L., and Blaser,M.J. (1993) Cloning and expression of a high-molecular-mass major antigen of Helicobacter pylori: evidence of linkage to cytotoxin production. *Infect Immun.* **61** (5), 1799-1809.

472. Odenbreit,S., Puls,J., Sedlmaier,B., Gerland,E., Fischer,W., and Haas,R. (2000) Translocation of Helicobacter pylori CagA into gastric epithelial cells by type IV secretion. *Science* **287** (5457), 1497-1500.

473. Selbach,M., Moese,S., Hurwitz,R., Hauck,C.R., Meyer,T.F., and Backert,S. (2003) The Helicobacter pylori CagA protein induces cortactin dephosphorylation and actin rearrangement by c-Src inactivation. *EMBO J.* **22** (3), 515-528.

474. Su,B., Ceponis,P.J., and Sherman,P.M. (2003) Cytoskeletal rearrangements in gastric epithelial cells in response to Helicobacter pylori infection. *J.Med.Microbiol.* **52** (Pt 10), 861-867.

475. Segal,E.D., Cha,J., Lo,J., Falkow,S., and Tompkins,L.S. (1999) Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by Helicobacter pylori. *Proc.Natl.Acad.Sci.U.S.A* **96** (25), 14559-14564.

476. Amieva,M.R., Vogelmann,R., Covacci,A., Tompkins,L.S., Nelson,W.J., and Falkow,S. (2003) Disruption of the epithelial apical-junctional complex by Helicobacter pylori CagA. *Science* **300** (5624), 1430-1434.

477. Mimuro,H., Suzuki,T., Tanaka,J., Asahi,M., Haas,R., and Sasakawa,C. (2002) Grb2 is a key mediator of helicobacter pylori CagA protein activities. *Mol.Cell* **10** (4), 745-755.

478. Tsutsumi,R., Higashi,H., Higuchi,M., Okada,M., and Hatakeyama,M. (2003) Attenuation of Helicobacter pylori CagA x SHP-2 signaling by interaction between CagA and C-terminal Src kinase. *J.Biol.Chem.* **278** (6), 3664-3670.

479. Yanai,A., Hirata,Y., Mitsuno,Y., Maeda,S., Shibata,W., Akanuma,M., Yoshida,H., Kawabe,T., and Omata,M. (2003) Helicobacter pylori induces antiapoptosis through buclear factor-kappaB activation. *J Infect Dis* **188** (11), 1741-1751.

480. Hamlet,A., Thoreson,A.C., Nilsson,O., Svennerholm,A.M., and Olbe,L. (1999) Duodenal Helicobacter pylori infection differs in cagA genotype between asymptomatic subjects and patients with duodenal ulcers. *Gastroenterology* **116** (2), 259-268.

481. Kato,K. and Ohara,S. (2000) Alteration of cellular turnover in Helicobacter pylori-infected gastric mucosa. *J.Gastroenterol.* **35** (4), 312-314.

482. Peek,R.M., Jr., Miller,G.G., Tham,K.T., Perez-Perez,G.I., Zhao,X., Atherton,J.C., and Blaser,M.J. (1995) Heightened inflammatory response and cytokine expression in vivo to cagA+ Helicobacter pylori strains. *Lab Invest* **73** (6), 760-770.

483. Yamaoka,Y., Kodama,T., Kita,M., Imanishi,J., Kashima,K., and Graham,D.Y. (1999) Relation between clinical presentation, Helicobacter pylori density, interleukin 1beta and 8 production, and cagA status. *Gut* **45** (6), 804-811.

484. Neu,B., Randlkofer,P., Neuhofer,M., Voland,P., Mayerhofer,A., Gerhard,M., Schepp,W., and Prinz,C. (2002) Helicobacter pylori induces apoptosis of rat gastric parietal cells. *Am.J.Physiol Gastrointest.Liver Physiol* **283** (2), G309-G318.

485. Day,A.S., Jones,N.L., Lynett,J.T., Jennings,H.A., Fallone,C.A., Beech,R., and Sherman,P.M. (2000) cagE is a virulence factor associated with Helicobacter pylori-induced duodenal ulceration in children. *J.Infect.Dis.* **181** (4), 1370-1375.

486. Atherton,J.C., Cao,P., Peek,R.M., Jr., Tummuru,M.K., Blaser,M.J., and Cover,T.L. (1995) Mosaicism in vacuolating cytotoxin alleles of Helicobacter pylori. Association of specific vacA types with cytotoxin production and peptic ulceration. *J.Biol.Chem.* **270** (30), 17771-17777.

487. Cover,T.L., Halter,S.A., and Blaser,M.J. (1992) Characterization of HeLa cell vacuoles induced by Helicobacter pylori broth culture supernatant. *Hum.Pathol.* **23** (9), 1004-1010.

488. Suzuki,J., Ohnsihi,H., Shibata,H., Wada,A., Hirayama,T., Iiri,T., Ueda,N., Kanamaru,C., Tsuchida,T., Mashima,H., Yasuda,H., and Fujita,T. (2001) Dynamin is involved in human epithelial cell vacuolation caused by the Helicobacter pylori-produced cytotoxin VacA. *J.Clin.Invest* **107** (3), 363-370.

489. Papini,E., De Bernard,M., Bugnoli,M., Milia,E., Rappuoli,R., and Montecucco,C. (1993) Cell vacuolization induced by Helicobacter pylori: inhibition by bafilomycins A1, B1, C1 and D. *FEMS Microbiol.Lett.* **113** (2), 155-159.

490. Papini,E., De Bernard,M., Milia,E., Bugnoli,M., Zerial,M., Rappuoli,R., and Montecucco,C. (1994) Cellular vacuoles induced by Helicobacter pylori originate from late endosomal compartments. *Proc.Natl.Acad.Sci.U.S.A* **91** (21), 9720-9724.

491. Papini,E., Gottardi,E., Satin,B., De Bernard,M., Massari,P., Telford,J., Rappuoli,R., Sato,S.B., and Montecucco,C. (1996) The vacuolar ATPase proton pump is present on intracellular vacuoles induced by Helicobacter pylori. *J.Med.Microbiol.* **45** (2), 84-89.

492. Catrenich,C.E. and Chestnut,M.H. (1992) Character and origin of vacuoles induced in mammalian cells by the cytotoxin of Helicobacter pylori. *J.Med.Microbiol.* **37** (6), 389-395.

493. Smoot,D.T., Resau,J.H., Earlington,M.H., Simpson,M., and Cover,T.L. (1996) Effects of Helicobacter pylori vacuolating cytotoxin on primary cultures of human gastric epithelial cells. *Gut* **39** (96), 795-799.

494. Iwamoto,H., Czajkowsky,D.M., Cover,T.L., Szabo,G., and Shao,Z. (1999) VacA from Helicobacter pylori: a hexameric chloride channel. *FEBS Lett.* **450** (1-2), 101-104.

495. Tombola,F., Morbiato,L., Del Giudice,G., Rappuoli,R., Zoratti,M., and Papini,E. (2001) The Helicobacter pylori VacA toxin is a urea permease that promotes urea diffusion across epithelia. *J.Clin.Invest* **108** (6), 929-937.

496. Papini,E., Satin,B., Norais,N., De Bernard,M., Telford,J.L., Rappuoli,R., and Montecucco,C. (1998) Selective increase of the permeability of polarized epithelial cell monolayers by Helicobacter pylori vacuolating toxin. *J Clin.Invest* **102** (4), 813-820.

497. Zheng,P.Y. and Jones,N.L. (2003) Helicobacter pylori strains expressing the vacuolating cytotoxin interrupt phagosome maturation in macrophages by recruiting and retaining TACO (coronin 1) protein. *Cell Microbiol.* **5** (1), 25-40.
Ref ID: 1909

498. Molinari,M., Salio,M., Galli,C., Norais,N., Rappuoli,R., Lanzavecchia,A., and Montecucco,C. (1998) Selective inhibition of Ii-dependent antigen presentation by Helicobacter pylori toxin VacA. *J.Exp.Med.* **187** (1), 135-140.

499. Gebert,B., Fischer,W., Weiss,E., Hoffmann,R., and Haas,R. (2003) Helicobacter pylori vacuolating cytotoxin inhibits T lymphocyte activation. *Science* **301** (5636), 1099-1102.

500. Beil,W., Enss,M.L., Muller,S., Obst,B., Sewing,K.F., and Wagner,S. (2000) Role of vacA and cagA in Helicobacter pylori inhibition of mucin synthesis in gastric mucous cells. *J.Clin.Microbiol.* **38** (6), 2215-2218.

501. Kobayashi,H., Kamiya,S., Suzuki,T., Kohda,K., Muramatsu,S., Kurumada,T., Ohta,U., Miyazawa,M., Kimura,N., Mutoh,N., Shirai,T., Takagi,A., Harasawa,S., Tani,N., and Miwa,T. (1996) The effect of Helicobacter pylori on gastric acid secretion by isolated parietal cells from a guinea pig. Association with production of vacuolating toxin by H. pylori. *Scand.J.Gastroenterol.* **31** (5), 428-433.

502. Pai,R., Cover,T.L., and Tarnawski,A.S. (1999) Helicobacter pylori vacuolating cytotoxin (VacA) disorganizes the cytoskeletal architecture of gastric epithelial cells. *Biochem.Biophys.Res.Commun.* **262** (1), 245-250.

503. Kuck,D., Kolmerer,B., Iking-Konert,C., Krammer,P.H., Stremmel,W., and Rudi,J. (2001) Vacuolating cytotoxin of Helicobacter pylori induces apoptosis in the human gastric epithelial cell line AGS. *Infect.Immun.* **69** (8), 5080-5087.

504. Cover,T.L., Krishna,U.S., Israel,D.A., and Peek,R.M., Jr. (2003) Induction of gastric epithelial cell apoptosis by Helicobacter pylori vacuolating cytotoxin. *Cancer Res.* **63** (5), 951-957.

505. Jones,A.D., Bacon,K.D., Jobe,B.A., Sheppard,B.C., Deveney,C.W., and Rutten,M.J. (2003) Helicobacter pylori induces apoptosis in Barrett's-derived esophageal adenocarcinoma cells. *J.Gastrointest.Surg.* **7** (1), 68-76.

506. Blaser,M.J. and Atherton,J.C. (2004) Helicobacter pylori persistence: biology and disease. *J Clin.Invest* **113** (3), 321-333.

507. Atherton,J.C. (1997) The clinical relevance of strain types of Helicobacter pylori. *Gut* **40** (6), 701-703.

508. Letley,D.P. and Atherton,J.C. (2000) Natural diversity in the N terminus of the mature vacuolating cytotoxin of Helicobacter pylori determines cytotoxin activity. *J.Bacteriol.* **182** (11), 3278-3280.

509. Xiang,Z., Censini,S., Bayeli,P.F., Telford,J.L., Figura,N., Rappuoli,R., and Covacci,A. (1995) Analysis of expression of CagA and VacA virulence factors in 43 strains of Helicobacter pylori reveals that clinical isolates can be divided into two major types and that CagA is not necessary for expression of the vacuolating cytotoxin. *Infect Immun.* **63** (1), 94-98.

510. Ge,Z. and Taylor,D.E. (1999) Contributions of genome sequencing to understanding the biology of Helicobacter pylori. *Annu.Rev.Microbiol* **53** 353-387.

511. Enroth,H., Kraaz,W., Engstrand,L., Nyren,O., and Rohan,T. (2000) Helicobacter pylori strain types and risk of gastric cancer: a case-control study. *Cancer Epidemiol.Biomarkers Prev.* **9** (9), 981-985.

512. Parsonnet,J., Friedman,G.D., Orentreich,N., and Vogelman,H. (1997) Risk for gastric cancer in people with CagA positive or CagA negative Helicobacter pylori infection. *Gut* **40** (3), 297-301.

513. Warburton,V.J., Everett,S., Mapstone,N.P., Axon,A.T., Hawkey,P., and Dixon,M.F. (1998) Clinical and histological associations of cagA and vacA genotypes in Helicobacter pylori gastritis. *J Clin.Pathol.* **51** (1), 55-61.

514. Semino-Mora,C., Doi,S.Q., Marty,A., Simko,V., Carlstedt,I., and Dubois,A. (2003) Intracellular and interstitial expression of Helicobacter pylori virulence genes in gastric precancerous intestinal metaplasia and adenocarcinoma. *J.Infect.Dis.* **187** (8), 1165-1177.

515. Nomura,A.M., Perez-Perez,G.I., Lee,J., Stemmermann,G., and Blaser,M.J. (2002) Relation between Helicobacter pylori cagA status and risk of peptic ulcer disease. *Am.J Epidemiol.* **155** (11), 1054-1059.

516. Quintero,E., Pizarro,M.A., Rodrigo,L., Pique,J.M., Lanas,A., Ponce,J., Mino,G., Gisbert,J., Jurado,A., Herrero,M.J., Jimenez,A., Torrado,J., Ponte,A., Diaz-de-Rojas,F., and Salido,E. (2005) Association of Helicobacter pylori-related distal gastric cancer with the HLA class II gene DQB10602 and cagA strains in a southern European population. *Helicobacter.* **10** (1), 12-21.

517. Koehler,C.I., Mues,M.B., Dienes,H.P., Kriegsmann,J., Schirmacher,P., and Odenthal,M. (2003) Helicobacter pylori genotyping in gastric adenocarcinoma and MALT lymphoma by multiplex PCR analyses of paraffin wax embedded tissues. *Mol.Pathol.* **56** (1), 36-42.

518. van Doorn,L.J., Figueiredo,C., Sanna,R., Plaisier,A., Schneeberger,P., de Boer,W., and Quint,W. (1998) Clinical relevance of the cagA, vacA, and iceA status of Helicobacter pylori. *Gastroenterology* **115** (1), 58-66.

519. van Doorn,L.J., Figueiredo,C., Rossau,R., Jannes,G., van Asbroek,M., Sousa,J.C., Carneiro,F., and Quint,W.G. (1998) Typing of Helicobacter pylori vacA gene and detection of cagA gene by PCR and reverse hybridization. *J.Clin.Microbiol.* **36** (5), 1271-1276.

520. Peek,R.M., Jr., Thompson,S.A., Donahue,J.P., Tham,K.T., Atherton,J.C., Blaser,M.J., and Miller,G.G. (1998) Adherence to gastric epithelial cells induces expression of a Helicobacter pylori gene, iceA, that is associated with clinical outcome. *Proc.Assoc.Am.Physicians* **110** (6), 531-544.

521. Wu,C.C., Chou,P.Y., Hu,C.T., Liu,Z.C., Lin,C.Y., Tseng,Y.H., and Lin,N.T. (2005) Clinical Relevance of the vacA, iceA, cagA, and flaA genes of Helicobacter pylori strains isolated in Eastern Taiwan. *J.Clin.Microbiol.* **43** (6), 2913-2915.

522. Ribeiro,M.L., Godoy,A.P., Benvengo,Y.H., Mendonca,S., and Pedrazzoli,J., Jr. (2003) Clinical relevance of the cagA, vacA and iceA genotypes of Helicobacter pylori in Brazilian clinical isolates. *FEMS Immunol.Med.Microbiol.* **36** (3), 181-185.

523. Evans,D.J., Jr., Evans,D.G., Takemura,T., Nakano,H., Lampert,H.C., Graham,D.Y., Granger,D.N., and Kvietys,P.R. (1995) Characterization of a Helicobacter pylori neutrophil-activating protein. *Infect.Immun.* **63** (6), 2213-2220.

524. Yoshida,N., Granger,D.N., Evans,D.J., Jr., Evans,D.G., Graham,D.Y., Anderson,D.C., Wolf,R.E., and Kvietys,P.R. (1993) Mechanisms involved in Helicobacter pylori-induced inflammation. *Gastroenterology* **105** (5), 1431-1440.

525. Kurose,I., Granger,D.N., Evans,D.J., Jr., Evans,D.G., Graham,D.Y., Miyasaka,M., Anderson,D.C., Wolf,R.E., Cepinskas,G., and Kvietys,P.R. (1994) Helicobacter pylori-induced microvascular protein leakage in rats: role of neutrophils, mast cells, and platelets. *Gastroenterology* **107** (1), 70-79.

526. Brisslert,M., Enarsson,K., Lundin,S., Karlsson,A., Kusters,J.G., Svennerholm,A.M., Backert,S., and Quiding-Jarbrink,M. (2005) Helicobacter pylori induce neutrophil transendothelial migration: role of the bacterial HP-NAP. *FEMS Microbiol.Lett.* **249** (1), 95-103.

527. Satin,B., Del Giudice,G., Della,B., V, Dusi,S., Laudanna,C., Tonello,F., Kelleher,D., Rappuoli,R., Montecucco,C., and Rossi,F. (2000) The neutrophil-activating protein (HP-NAP) of Helicobacter pylori is a protective antigen and a major virulence factor. *J.Exp.Med.* **191** (9), 1467-1476.

528. Namavar,F., Sparrius,M., Veerman,E.C., Appelmelk,B.J., and Vandenbroucke-Grauls,C.M. (1998) Neutrophil-activating protein mediates adhesion of Helicobacter pylori to sulfated carbohydrates on high-molecular-weight salivary mucin. *Infect.Immun.* **66** (2), 444-447.

529. Aspinall,G.O., Monteiro,M.A., Pang,H., Walsh,E.J., and Moran,A.P. (1996) Lipopolysaccharide of the Helicobacter pylori type strain NCTC 11637 (ATCC 43504): structure of the O antigen chain and core oligosaccharide regions. *Biochemistry* **35** (7), 2489-2497.

530. Aspinall,G.O. and Monteiro,M.A. (1996) Lipopolysaccharides of Helicobacter pylori strains P466 and MO19: structures of the O antigen and core oligosaccharide regions. *Biochemistry* **35** (7), 2498-2504.

531. Mollicone,R.J., Bara,J., LePendu,J., and Oriol,R. (1985) Immunohistologic pattern of type 1 (Le^a, Le^b) and type 2 (X, Y, H) blood group-related antigens in the human pyloric and duodenal mucosa. *Lab.Investig.* (53), 219-227.

532. Sakamoto,S., Watanabe,T., Tokumaru,T., Takagi,H., Nakazato,H., and Lloyd,K.O. (1989) Expression of Lewisa, Lewisb, Lewisx, Lewisy, siayl-Lewisa, and sialyl-Lewisx blood group antigens in human gastric carcinoma and in normal gastric tissue. *Cancer Res.* **49** (3), 745-752.

533. Moran,A.P., Prendergast,M.M., and Appelmelk,B.J. (1996) Molecular mimicry of host structures by bacterial lipopolysaccharides and its contribution to disease. *FEMS Immunol.Med.Microbiol.* **16** (2), 105-115.

534. Wirth,H.P., Yang,M., Karita,M., and Blaser,M.J. (1996) Expression of the human cell surface glycoconjugates Lewis x and Lewis y by Helicobacter pylori isolates is related to cagA status. *Infect.Immun.* **64** (11), 4598-4605.

535. Simoons-Smit,I.M., Appelmelk,B.J., Verboom,T., Negrini,R., Penner,J.L., Aspinall,G.O., Moran,A.P., Fei,S.F., Shi,B.S., Rudnica,W., Savio,A., and de Graaff,J. (1996) Typing of Helicobacter pylori with monoclonal antibodies against Lewis antigens in lipopolysaccharide. *J.Clin.Microbiol.* **34** (9), 2196-2200.

536. Moran,A.P. (1996) The role of lipopolysaccharide in Helicobacter pylori pathogenesis. *Aliment.Pharmacol.Ther.* **10 Suppl 1** 39-50.

537. Monteiro,M.A., Rasko,D., Taylor,D.E., and Perry,M.B. (1998) Glucosylated N-acetyllactosamine O-antigen chain in the lipopolysaccharide from Helicobacter pylori strain UA861. *Glycobiology* **8** (1), 107-112.

538. Eaton,K.A., Logan,S.M., Baker,P.E., Peterson,R.A., Monteiro,M.A., and Altman,E. (2004) Helicobacter pylori with a truncated lipopolysaccharide O chain fails to induce gastritis in SCID mice injected with splenocytes from wild-type C57BL/6J mice. *Infect.Immun.* **72** (7), 3925-3931.

539. Slomiany,B.L. and Slomiany,A. (2005) Up-regulation in endothelin-1 by Helicobacter pylori lipopolysaccharide interferes with gastric mucin synthesis via epidermal growth factor receptor transactivation. *Scand.J.Gastroenterol.* **40** (8), 921-928.

540. Khamri,W., Moran,A.P., Worku,M.L., Karim,Q.N., Walker,M.M., Annuk,H., Ferris,J.A., Appelmelk,B.J., Eggleton,P., Reid,K.B., and Thursz,M.R. (2005) Variations in Helicobacter pylori lipopolysaccharide to evade the innate immune component surfactant protein D. *Infect.Immun.* **73** (11), 7677-7686.

541. Kuipers,E.J., Thijs,J.C., and Festen,H.P. (1995) The prevalence of Helicobacter pylori in peptic ulcer disease. *Aliment.Pharmacol.Ther.* **9 Suppl 2** 59-69.

542. Eid,R. and Moss,S.F. (2002) Helicobacter pylori infection and the development of gastric cancer. *N.Engl.J Med.* **346** (1), 65-67.

543. Forman,D., Newell,D.G., Fullerton,F., Yarnell,J.W., Stacey,A.R., Wald,N., and Sitas,F. (1991) Association between infection with Helicobacter pylori and risk of gastric cancer: evidence from a prospective investigation. *BMJ* **302** (6788), 1302-1305.

544. Fox,J.G. (1998) Review article: Helicobacter species and in vivo models of gastrointestinal cancer. *Aliment.Pharmacol.Ther.* **12 Suppl 1** 37-60.

545. Parsonnet,J. and Isaacson,P.G. (2004) Bacterial infection and MALT lymphoma. *N.Engl.J Med.* **350** (3), 213-215.
546. Uemura,N., Okamoto,S., Yamamoto,S., Matsumura,N., Yamaguchi,S., Yamakido,M., Taniyama,K., Sasaki,N., and Schlemper,R.J. (2001) Helicobacter pylori infection and the development of gastric cancer. *N.Engl.J Med.* **345** (11), 784-789.

547. Wotherspoon,A.C. (1998) Helicobacter pylori infection and gastric lymphoma. *Br.Med.Bull.* **54** (1), 79-85.

548. Wotherspoon,A.C. (1998) Gastric lymphoma of mucosa-associated lymphoid tissue and Helicobacter pylori. *Annu.Rev.Med.* **49** 289-299.

549. Xu,H.T., Zhao,Y.F., Lian,Z.X., Fan,B.L., Zhao,Z.H., Yu,S.Y., Dai,Y.P., Wang,L.L., Niu,H.L., Li,N., Hammarstrom,L., Boren,T., and Sjostrom,R. (2004) Effects of fucosylated milk of goat and mouse on Helicobacter pylori binding to Lewis b antigen. *World J.Gastroenterol.* **10** (14), 2063-2066.

550. Isaacson,P.G. (2005) Update on MALT lymphomas. *Best.Pract.Res.Clin.Haematol.* **18** (1), 57-68.

551. McColl,K.E., el Omar,E., and Gillen,D. (1998) Interactions between H. pylori infection, gastric acid secretion and anti-secretory therapy. *Br.Med.Bull.* **54** (1), 121-138.

552. Kidd,M., Modlin,I.M., and Tang,L.H. (1998) Gastrin and the enterochromaffin-like cell: an acid update. *Dig.Surg.* **15** (3), 209-217.

553. Makhlouf,G.M. and Schubert,M.L. (1990) Gastric somatostatin: a paracrine regulator of acid secretion. *Metabolism* **39** (9 Suppl 2), 138-142.

554. Lee,A. (1994) The microbiology and epidemiology of Helicobacter pylori infection. *Scand.J.Gastroenterol.Suppl* **201** 2-6.

555. Blaser,M.J. (1987) Gastric Campylobacter-like organisms, gastritis, and peptic ulcer disease. *Gastroenterology* **93** (2), 371-383.

556. Sipponen,P., Hyvarinen,H., and Siurala,M. (1996) H. pylori corpus gastritis--relation to acid output. *J.Physiol Pharmacol.* **47** (1), 151-159.
557. Sipponen,P., Kekki,M., Seppala,K., and Siurala,M. (1996) The relationships between chronic gastritis and gastric acid secretion. *Aliment.Pharmacol.Ther.* **10 Suppl 1** 103-118.

558. Akada,J.K., Ogura,K., Dailidiene,D., Dailide,G., Cheverud,J.M., and Berg,D.E. (2003) Helicobacter pylori tissue tropism: mouse-colonizing strains can target different gastric niches. *Microbiology* **149** (Pt 7), 1901-1909.

559. Syder,A.J., Oh,J.D., Guruge,J.L., O'Donnell,D., Karlsson,M., Mills,J.C., Bjorkholm,B.M., and Gordon,J.I. (2003) The impact of parietal cells on Helicobacter pylori tropism and host pathology: an analysis using gnotobiotic normal and transgenic mice. *Proc.Natl.Acad.Sci.U.S.A* **100** (6), 3467-3472.

560. Hamlet,A. and Olbe,L. (1996) The influence of Helicobacter pylori infection on postprandial duodenal acid load and duodenal bulb pH in humans. *Gastroenterology* **111** (2), 391-400.

561. Koskenpato,J., Farkkila,M., and Sipponen,P. (2002) Helicobacter pylori and different topographic types of gastritis: treatment response after successful eradication therapy in functional dyspepsia. *Scand.J.Gastroenterol.* **37** (7), 778-784.

562. Moss,S.F., Legon,S., Bishop,A.E., Polak,J.M., and Calam,J. (1992) Effect of Helicobacter pylori on gastric somatostatin in duodenal ulcer disease. *Lancet* **340** (8825), 930-932.

563. Lamers,C.B. (1992) Gastric secretory abnormalities in duodenal ulcer: primary or secondary to Helicobacter pylori infection? *Scand.J Gastroenterol.Suppl* **194** 99-103.

564. Levi,S., Beardshall,K., Desa,L.A., and Calam,J. (1989) Campylobacter pylori, gastrin, acid secretion, and duodenal ulcers. *Lancet* **2** (8663), 613.

565. Levi,S., Beardshall,K., Haddad,G., Playford,R., Ghosh,P., and Calam,J. (1989) Campylobacter pylori and duodenal ulcers: the gastrin link. *Lancet* **1** (8648), 1167-1168.

566. Chowers,M.Y., Keller,N., Bar-Meir,S., and Chowers,Y. (2002) A defined human gastrin sequence stimulates the growth of Helicobacter pylori. *FEMS Microbiol Lett.* **217** (2), 231-236.

567. Beales,I.L. (1998) Effect of cytokines on acid secretion and gastrin secretion in Helicobacter pylori infection and aspirin-induced gastritis. *Scand.J Gastroenterol.* **33** (11), 1230-1232.

568. Beales,I.L. and Calam,J. (1998) Interleukin 1 beta and tumour necrosis factor alpha inhibit acid secretion in cultured rabbit parietal cells by multiple pathways. *Gut* **42** (2), 227-234.

569. El Omar,E.M., Penman,I.D., Ardill,J.E., Chittajallu,R.S., Howie,C., and McColl,K.E. (1995) Helicobacter pylori infection and abnormalities of acid secretion in patients with duodenal ulcer disease. *Gastroenterology* **109** (3), 681-691.

570. Gillen,D., El Omar,E.M., Wirz,A.A., Ardill,J.E., and McColl,K.E. (1998) The acid response to gastrin distinguishes duodenal ulcer patients from Helicobacter pylori-infected healthy subjects. *Gastroenterology* **114** (1), 50-57.

571. Tuo,B.G., Sellers,Z.M., Smith,A.J., Barrett,K.E., Isenberg,J.I., and Dong,H. (2004) A role for CagA/VacA in Helicobacter pylori inhibition of murine duodenal mucosal bicarbonate secretion. *Dig.Dis Sci.* **49** (11-12), 1845-1852.

572. Walker,M.M. and Crabtree,J.E. (1998) Helicobacter pylori infection and the pathogenesis of duodenal ulceration. *Ann.N.Y.Acad.Sci.* **859** 96-111.

573. Khulusi,S., Badve,S., Patel,P., Lloyd,R., Marrero,J.M., Finlayson,C., Mendall,M.A., and Northfield,T.C. (1996) Pathogenesis of gastric metaplasia of the human duodenum: role of Helicobacter pylori, gastric acid, and ulceration. *Gastroenterology* **110** (2), 452-458.

574. Ohkusa,T., Okayasu,I., Miwa,H., Ohtaka,K., Endo,S., and Sato,N. (2003) Helicobacter pylori infection induces duodenitis and superficial duodenal ulcer in Mongolian gerbils. *Gut* **52** (6), 797-803.

575. Lazebnik,L.B., Arbuzova,V.G., Sokolova,G.N., Astaf'eva,O.V., Petrakov,A.V., Nilova,T.V., and Chikunova,B.Z. (2002) [Role of stress in the etiopathogenesis of duodenal ulcer in young patients]. *Eksp.Klin.Gastroenterol.* (5), 30-37.

576. Davidovic,M., Svorcan,P., Milanovic,P., Antovic,A., and Milosevic,D. (2005) Specifics of Helicobacter pylori infection/NSAID effects in the elderly. *Rom.J Gastroenterol.* **14** (3), 253-258.

577. Greenwald,D.A. (2004) Aging, the gastrointestinal tract, and risk of acid-related disease. *Am.J Med.* **117 Suppl 5A** 8S-13S.

578. Lunet,N. and Barros,H. (2003) Helicobacter pylori infection and gastric cancer: facing the enigmas. *Int.J.Cancer* **106** (6), 953-960.

579. Endoh,K. and Leung,F.W. (1994) Effects of smoking and nicotine on the gastric mucosa: a review of clinical and experimental evidence. *Gastroenterology* **107** (3), 864-878.

580. Huang,J.Q., Sridhar,S., and Hunt,R.H. (2002) Role of Helicobacter pylori infection and non-steroidal anti-inflammatory drugs in peptic-ulcer disease: a meta-analysis. *Lancet* **359** (9300), 14-22.

581. Kozol,R.A. and Dekhne,N. (1994) Helicobacter pylori and the pathogenesis of duodenal ulcer. *J.Lab Clin.Med.* **124** (5), 623-626.

582. Correa,P. (1992) Human gastric carcinogenesis: a multistep and multifactorial process--First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. *Cancer Res.* **52** (24), 6735-6740.

583. Sipponen,P., Hyvarinen,H., Seppala,K., and Blaser,M.J. (1998) Review article: Pathogenesis of the transformation from gastritis to malignancy. *Aliment.Pharmacol.Ther.* **12 Suppl 1** 61-71.

584. Leach,S.A., Thompson,M., and Hill,M. (1987) Bacterially catalysed N-nitrosation reactions and their relative importance in the human stomach. *Carcinogenesis* **8** (12), 1907-1912.

585. Naylor,G. and Axon,A. (2003) Role of bacterial overgrowth in the stomach as an additional risk factor for gastritis. *Can.J Gastroenterol.* **17 Suppl B** 13B-17B.

586. Williams,C. and McColl,K.E. (2006) Review article: proton pump inhibitors and bacterial overgrowth. *Aliment.Pharmacol.Ther.* **23** (1), 3-10.

587. Yang,Y.L., Xu,B., Song,Y.G., and Zhang,W.D. (2003) Overexpression of c-fos in Helicobacter pylori-induced gastric precancerosis of Mongolian gerbil. *World J Gastroenterol.* **9** (3), 521-524.

588. Zhuang,X.Q. and Lin,S.R. (2000) Research of Helicobacter pylori infection in precancerous gastric lesions. *World J Gastroenterol.* **6** (3), 428-429.

589. El Omar,E.M., Oien,K., El Nujumi,A., Gillen,D., Wirz,A., Dahill,S., Williams,C., Ardill,J.E., and McColl,K.E. (1997) Helicobacter pylori infection and chronic gastric acid hyposecretion. *Gastroenterology* **113** (1), 15-24.

590. El Omar,E.M., Carrington,M., Chow,W.H., McColl,K.E., Bream,J.H., Young,H.A., Herrera,J., Lissowska,J., Yuan,C.C., Rothman,N., Lanyon,G., Martin,M., Fraumeni,J.F., Jr., and Rabkin,C.S. (2000) Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* **404** (6776), 398-402.

591. Knight,T., Wyatt,J., Wilson,A., Greaves,S., Newell,D., Hengels,K., Corlett,M., Webb,P., Forman,D., and Elder,J. (1996) Helicobacter pylori gastritis and serum pepsinogen levels in a healthy population: development of a biomarker strategy for gastric atrophy in high risk groups. *Br.J Cancer* **73** (6), 819-824.

592. Wang,T.C., Dangler,C.A., Chen,D., Goldenring,J.R., Koh,T., Raychowdhury,R., Coffey,R.J., Ito,S., Varro,A., Dockray,G.J., and Fox,J.G. (2000) Synergistic interaction between hypergastrinemia and Helicobacter infection in a mouse model of gastric cancer. *Gastroenterology* **118** (1), 36-47.

593. Kodama,K., Sumii,K., Kawano,M., Kido,T., Nojima,K., Sumii,M., Haruma,K., Yoshihara,M., and Chayama,K. (2003) Gastric juice nitrite and vitamin C in patients with gastric cancer and atrophic gastritis: is low acidity solely responsible for cancer risk? *Eur.J Gastroenterol.Hepatol.* **15** (9), 987-993.

594. Shiotani,A., Yanaoka,K., Iguchi,M., Saika,A., Itoh,H., and Nishioka,S. (1999) Helicobacter pylori infection reduces intraluminal nitric oxide in humans. *J Gastroenterol.* **34** (6), 668-674.

595. Farinati,F., Della,L.G., Cardin,R., Molari,A., Plebani,M., Rugge,M., Di Mario,F., and Naccarato,R. (1996) Gastric antioxidant, nitrites, and mucosal lipoperoxidation in chronic gastritis and Helicobacter pylori infection. *J Clin.Gastroenterol.* **22** (4), 275-281.

596. Tari,A., Kodama,K., Kitadai,Y., Ohta,M., Sumii,K., and Kajiyama,G. (2003) Is apoptosis in antral mucosa correlated with serum nitrite concentration in Japanese Helicobacter pylori-infected patients? *J.Gastroenterol.Hepatol.* **18** (5), 498-504.

597. Tari,A., Kodama,K., Sumii,M., Tani,H., Sumii,K., and Chayama,K. (2003) Does intragastric nitrite concentration reflect gastric carcinogenesis in Japanese Helicobacter pylori-infected patients? *Dig.Dis.Sci.* **48** (9), 1730-1736.

598. Stolte,M. (1992) Helicobacter pylori gastritis and gastric MALT-lymphoma. *Lancet* **339** (8795), 745-746.

599. Hussell,T., Isaacson,P.G., Crabtree,J.E., and Spencer,J. (1996) Helicobacter pylori-specific tumour-infiltrating T cells provide contact dependent help for the growth of malignant B cells in low-grade gastric lymphoma of mucosa-associated lymphoid tissue. *J Pathol.* **178** (2), 122-127.

600. Wohlfart,S., Sebinger,D., Gruber,P., Buch,J., Polgar,D., Krupitza,G., Rosner,M., Hengstschlager,M., Raderer,M., Chott,A., and Mullauer,L. (2004) FAS (CD95) mutations are rare in gastric MALT lymphoma but occur more frequently in primary gastric diffuse large B-cell lymphoma. *Am.J Pathol.* **164** (3), 1081-1089.

601. Ohmae,T., Hirata,Y., Maeda,S., Shibata,W., Yanai,A., Ogura,K., Yoshida,H., Kawabe,T., and Omata,M. (2005) Helicobacter pylori activates NF-kappaB via the alternative pathway in B lymphocytes. *J Immunol.* **175** (11), 7162-7169.

602. Toro,R.C., Garcia-Samaniego,J., Casado,F., I, Rubio,A.M., and Baquero,M.M. (2003) [Clinical importance of the CagA and VacA proteins and of the host factores in the development of peptic ulcer in patients infected by Helicobacter pylori]. *Rev.Clin.Esp.* **203** (9), 430-433.

603. Zambon,C.F., Basso,D., Navaglia,F., Germano,G., Gallo,N., Milazzo,M., Greco,E., Fogar,P., Mazza,S., Di Mario,F., Basso,G., Rugge,M., and Plebani,M. (2002) Helicobacter pylori virulence genes and host IL-1RN and IL-1beta genes interplay in favouring the development of peptic ulcer and intestinal metaplasia. *Cytokine* **18** (5), 242-251.

604. Gerhard,M., Lehn,N., Neumayer,N., Boren,T., Rad,R., Schepp,W., Miehlke,S., Classen,M., and Prinz,C. (1999) Clinical relevance of the Helicobacter pylori gene for blood-group antigen-binding adhesin. *Proc.Natl.Acad.Sci.U.S.A* **96** (22), 12778-12783.

605. Zambon,C.F., Navaglia,F., Basso,D., Rugge,M., and Plebani,M. (2003) Helicobacter pylori babA2, cagA, and s1 vacA genes work synergistically in causing intestinal metaplasia. *J.Clin.Pathol.* **56** (4), 287-291.

606. Day,A.S. and Sherman,P.M. (2000) Helicobacter pylori infection, host genes, and disease outcome. *Pediatr.Res.* **47** (6), 703.

607. De Gusmao,V.R., Nogueira,M.E., Magalhaes Queiroz,D.M., Aguiar,R.G., Camargos Rocha,A.M., Ramadan Ashour,A.A., and Teles Carvalho,A.S. (2000) vacA genotypes in Helicobacter pylori strains isolated from children with and without duodenal ulcer in Brazil. *J.Clin.Microbiol.* **38** (8), 2853-2857.

608. Logan,R.P. (1996) Adherence of Helicobacter pylori. *Aliment.Pharmacol.Ther.* **10 Suppl 1** 3-15.

609. Evans,D.J., Jr. and Evans,D.G. (2000) Helicobacter pylori adhesins: review and perspectives. *Helicobacter.* **5** (4), 183-195.

610. Kawakubo,M., Ito,Y., Okimura,Y., Kobayashi,M., Sakura,K., Kasama,S., Fukuda,M.N., Fukuda,M., Katsuyama,T., and Nakayama,J. (2004) Natural antibiotic function of a human gastric mucin against Helicobacter pylori infection. *Science* **305** (5686), 1003-1006.

611. Clyne,M., Dillon,P., Daly,S., O'Kennedy,R., May,F.E., Westley,B.R., and Drumm,B. (2004) Helicobacter pylori interacts with the human single-domain trefoil protein TFF1. *Proc.Natl.Acad.Sci.U.S.A* **101** (19), 7409-7414.

612. Van de Bovenkamp,J.H., Mahdavi,J., Korteland-Van Male,A.M., Buller,H.A., Einerhand,A.W., Boren,T., and Dekker,J. (2003) The MUC5AC glycoprotein is the primary receptor for Helicobacter pylori in the human stomach. *Helicobacter.* **8** (5), 521-532.

613. Wright,N.A., Hoffmann,W., Otto,W.R., Rio,M.C., and Thim,L. (1997) Rolling in the clover: trefoil factor family (TFF)-domain peptides, cell migration and cancer. *FEBS Lett.* **408** (2), 121-123.

614. Dignass,A., Lynch-Devaney,K., Kindon,H., Thim,L., and Podolsky,D.K. (1994) Trefoil peptides promote epithelial migration through a transforming growth factor beta-independent pathway. *J.Clin.Invest* **94** (1), 376-383.

615. Rio,M.C., Bellocq,J.P., Daniel,J.Y., Tomasetto,C., Lathe,R., Chenard,M.P., Batzenschlager,A., and Chambon,P. (1988) Breast cancer-associated pS2 protein: synthesis and secretion by normal stomach mucosa. *Science* **241** (4866), 705-708.

616. Hanby,A.M., Poulsom,R., Singh,S., Elia,G., Jeffery,R.E., and Wright,N.A. (1993) Spasmolytic polypeptide is a major antral peptide: distribution of the trefoil peptides human spasmolytic polypeptide and pS2 in the stomach. *Gastroenterology* **105** (4), 1110-1116.

617. Podolsky,D.K., Lynch-Devaney,K., Stow,J.L., Oates,P., Murgue,B., De Beaumont,M., Sands,B.E., and Mahida,Y.R. (1993) Identification of human intestinal trefoil factor. Goblet cell-specific expression of a peptide targeted for apical secretion. *J Biol.Chem.* **268** (16), 12230.

618. Van den Brink,G.R., Tytgat,K.M., Van der Hulst,R.W., Van der Loos,C.M., Einerhand,A.W., Buller,H.A., and Dekker,J. (2000) H pylori colocalises with MUC5AC in the human stomach. *Gut* **46** (5), 601-607.

619. Genta,R.M., Gurer,I.E., Graham,D.Y., Krishnan,B., Segura,A.M., Gutierrez,O., Kim,J.G., and Burchette,J.L., Jr. (1996) Adherence of Helicobacter pylori to areas of incomplete intestinal metaplasia in the gastric mucosa. *Gastroenterology* **111** (5), 1206-1211.

620. Linden,S., Nordman,H., Hedenbro,J., Hurtig,M., Boren,T., and Carlstedt,I. (2002) Strain- and blood group-dependent binding of Helicobacter pylori to human gastric MUC5AC glycoforms. *Gastroenterology* **123** (6), 1923-1930.

621. Mahdavi,J., Sonden,B., Hurtig,M., Olfat,F.O., Forsberg,L., Roche,N., Angstrom,J., Larsson,T., Teneberg,S., Karlsson,K.A., Altraja,S., Wadstrom,T., Kersulyte,D., Berg,D.E., Dubois,A., Petersson,C., Magnusson,K.E., Norberg,T., Lindh,F., Lundskog,B.B., Arnqvist,A., Hammarstrom,L., and Boren,T. (2002) Helicobacter pylori SabA adhesin in persistent infection and chronic inflammation. *Science* **297** (5581), 573-578.

622. Iver,D., Arnqvist,A., Ogren,J., Frick,I.M., Kersulyte,D., Incecik,E.T., Berg,D.E., Covacci,A., Engstrand,L., and Boren,T. (1998) Helicobacter pylori adhesin binding fucosylated histo-blood group antigens revealed by retagging. *Science* **279** (5349), 373-377.

623. Yuan,M., Itzkowitz,S.H., Palekar,A., Shamsuddin,A.M., Phelps,P.C., Trump,B.F., and Kim,Y.S. (1985) Distribution of blood group antigens A, B, H, Lewisa, and Lewisb in human normal, fetal, and malignant colonic tissue. *Cancer Res.* **45** (9), 4499-4511.

624. Lemieux,R.U., Baker,D.A., Weinstein,W.M., and Switzer,C.M. (1981) Artificial antigens. Antibody preparations for the localization of Lewis determinants in tissues. *Biochemistry* **20** (1), 199-205.

625. Jass,J.R. and Roberton,A.M. (1994) Colorectal mucin histochemistry in health and disease: a critical review. *Pathol.Int.* **44** (7), 487-504.

626. Nishihara,S., Hiraga,T., Ikehara,Y., Kudo,T., Iwasaki,H., Morozumi,K., Akamatsu,S., Tachikawa,T., and Narimatsu,H. (1999) Molecular mechanisms of expression of Lewis b antigen and other type I Lewis antigens in human colorectal cancer. *Glycobiology* **9** (6), 607-616.

627. Soejima,M. and Koda,Y. (2005) Molecular mechanisms of Lewis antigen expression. *Leg.Med.(Tokyo)* **7** (4), 266-269.

628. Sakamoto,J., Yin,B.W., and Lloyd,K.O. (1984) Analysis of the expression of H, Lewis, X, Y and precursor blood group determinants in saliva and red cells using a panel of mouse monoclonal antibodies. *Mol.Immunol.* **21** (11), 1093-1098.

629. Lin,C.W., Chang,Y.S., Wu,S.C., and Cheng,K.S. (1998) Helicobacter pylori in gastric biopsies of Taiwanese patients with gastroduodenal diseases. *Jpn.J Med.Sci.Biol.* **51** (1), 13-23.

630. Boren,T., Normark,S., and Falk,P. (1994) Helicobacter pylori: molecular basis for host recognition and bacterial adherence. *Trends Microbiol.* **2** (7), 221-228.

631. Kanbay,M., Gur,G., Arslan,H., Yilmaz,U., and Boyacioglu,S. (2005) The relationship of ABO blood group, age, gender, smoking, and Helicobacter pylori infection. *Dig.Dis Sci.* **50** (7), 1214-1217.

632. Mentis,A., Blackwell,C.C., Weir,D.M., Spiliadis,C., Dailianas,A., and Skandalis,N. (1991) ABO blood group, secretor status and detection of Helicobacter pylori among patients with gastric or duodenal ulcers. *Epidemiol.Infect.* **106** (2), 221-229.

633. Clarke,C.A., Edwards,J.W., Haddock,D.R., Howel-Evans,A.W., Mcconnell,R.B., and Sheppard,P.M. (1956) ABO blood groups and secretor character in duodenal ulcer; population and sibship studies. *Br.Med.J* **12** (4995), 725-731.

634. Aspholm-Hurtig,M., Dailide,G., Lahmann,M., Kalia,A., Ilver,D., Roche,N., Vikstrom,S., Sjostrom,R., Linden,S., Backstrom,A., Lundberg,C., Arnqvist,A., Mahdavi,J., Nilsson,U.J., Velapatino,B., Gilman,R.H., Gerhard,M., Alarcon,T., Lopez-Brea,M., Nakazawa,T., Fox,J.G., Correa,P., Dominguez-Bello,M.G., Perez-Perez,G.I., Blaser,M.J., Normark,S., Carlstedt,I., Oscarson,S., Teneberg,S., Berg,D.E., and Boren,T. (2004) Functional adaptation of BabA, the H. pylori ABO blood group antigen binding adhesin. *Science* **305** (5683), 519-522.

635. Miller-Podraza,H., Milh,M.A., Bergstrom,J., and Karlsson,K.A. (1996) Recognition of glycoconjugates by Helicobacter pylori: an apparently high-affinity binding of human polyglycosylceramides, a second sialic acid-based specificity. *Glycoconj.J.* **13** (3), 453-460.

636. Miller-Podraza,H., Bergstrom,J., Milh,M.A., and Karlsson,K.A. (1997) Recognition of glycoconjugates by Helicobacter pylori. Comparison of two sialic acid-dependent specificities based on haemagglutination and binding to human erythrocyte glycoconjugates. *Glycoconj.J.* **14** (4), 467-471.

637. Edwards,N.J., Monteiro,M.A., Faller,G., Walsh,E.J., Moran,A.P., Roberts,I.S., and High,N.J. (2000) Lewis X structures in the O antigen side-chain promote adhesion of Helicobacter pylori to the gastric epithelium. *Mol.Microbiol.* **35** (6), 1530-1539.

638. Mahdavi,J., Boren,T., Vandenbroucke-Grauls,C., and Appelmelk,B.J. (2003) Limited role of lipopolysaccharide Lewis antigens in adherence of Helicobacter pylori to the human gastric epithelium. *Infect Immun.* **71** (5), 2876-2880.

639. Valkonen,K.H., Wadstrom,T., and Moran,A.P. (1994) Interaction of lipopolysaccharides of Helicobacter pylori with basement membrane protein laminin. *Infect.Immun.* **62** (9), 3640-3648.

640. Taylor,D.E., Rasko,D.A., Sherburne,R., Ho,C., and Jewell,L.D. (1998) Lack of correlation between Lewis antigen expression by Helicobacter pylori and gastric epithelial cells in infected patients. *Gastroenterology* **115** (5), 1113-1122.

641. Trust,T.J., Doig,P., Emody,L., Kienle,Z., Wadstrom,T., and O'Toole,P. (1991) High-affinity binding of the basement membrane proteins collagen type IV and laminin to the gastric pathogen Helicobacter pylori. *Infect.Immun.* **59** (12), 4398-4404.

642. Valkonen,K.H., Ringner,M., Ljungh,A., and Wadstrom,T. (1993) High-affinity binding of laminin by Helicobacter pylori: evidence for a lectin-like interaction. *FEMS Immunol.Med.Microbiol.* **7** (1), 29-37.

643. Knibbs,R.N., Perini,F., and Goldstein,I.J. (1989) Structure of the major concanavalin A reactive oligosaccharides of the extracellular matrix component laminin. *Biochemistry* **28** (15), 6379-6392.

644. Guzman-Murillo,M.A. and Ascencio,F. (2000) Anti-adhesive activity of sulphated exopolysaccharides of microalgae on attachment of red sore disease-associated bacteria and helicobacter pylori to tissue culture cells. *Lett.Appl.Microbiol.* **30** (6), 473-478.

645. Guzman-Murillo,M.A., Ruiz-Bustos,E., Ho,B., and Ascencio,F. (2001) Involvement of the heparan sulphate-binding proteins of Helicobacter pylori in its adherence to HeLa S3 and Kato III cell lines. *J.Med.Microbiol.* **50** (4), 320-329.

646. Ruiz-Bustos,E., Ochoa,J.L., Wadstrom,T., and Ascencio,F. (2001) Isolation and characterisation of putative adhesins from Helicobacter pylori with affinity for heparan sulphate proteoglycan. *J.Med.Microbiol.* **50** (3), 215-222.

647. Kamisago,S., Iwamori,M., Tai,T., Mitamura,K., Yazaki,Y., and Sugano,K. (1996) Role of sulfatides in adhesion of Helicobacter pylori to gastric cancer cells. *Infect.Immun.* **64** (2), 624-628.

648. Ascencio,F., Fransson,L.A., and Wadstrom,T. (1993) Affinity of the gastric pathogen Helicobacter pylori for the N-sulphated glycosaminoglycan heparan sulphate. *J.Med.Microbiol.* **38** (4), 240-244.

649. Utt,M. and Wadstrom,T. (1997) Identification of heparan sulphate binding surface proteins of Helicobacter pylori: inhibition of heparan sulphate binding with sulphated carbohydrate polymers. *J.Med.Microbiol.* **46** (7), 541-546.

650. Ringner,M., Paulsson,M., and Wadstrom,T. (1992) Vitronectin binding by Helicobacter pylori. *FEMS Microbiol.Immunol.* **5** (4), 219-224.

651. Pantzar,M., Ljungh,A., and Wadstrom,T. (1998) Plasminogen binding and activation at the surface of Helicobacter pylori CCUG 17874. *Infect.Immun.* **66** (10), 4976-4980.

652. Hirmo,S., Kelm,S., Schauer,R., Nilsson,B., and Wadstrom,T. (1996) Adhesion of Helicobacter pylori strains to alpha-2,3-linked sialic acids. *Glycoconj.J.* **13** (6), 1005-1011.

653. Aspinall,G.O., Monteiro,M.A., Moran,A.P., Pang,H., Penner,J.L., and Shaver,R.T. (1995) Lipopolysaccharides from Helicobacter pylori. *Prog.Clin.Biol.Res.* **392** 93-101.

654. Monteiro,M.A., Chan,K.H., Rasko,D.A., Taylor,D.E., Zheng,P.Y., Appelmelk,B.J., Wirth,H.P., Yang,M., Blaser,M.J., Hynes,S.O., Moran,A.P., and Perry,M.B. (1998) Simultaneous expression of type 1 and type 2 Lewis blood group antigens by Helicobacter pylori lipopolysaccharides. Molecular mimicry between h. pylori lipopolysaccharides and human gastric epithelial cell surface glycoforms. *J Biol.Chem.* **273** (19), 11533-11543.

655. Teneberg,S., Miller-Podraza,H., Lampert,H.C., Evans,D.J., Jr., Evans,D.G., Danielsson,D., and Karlsson,K.A. (1997) Carbohydrate binding specificity of the neutrophil-activating protein of Helicobacter pylori. *J.Biol.Chem.* **272** (30), 19067-19071.

656. Saitoh,T., Natomi,H., Zhao,W.L., Okuzumi,K., Sugano,K., Iwamori,M., and Nagai,Y. (1991) Identification of glycolipid receptors for Helicobacter pylori by TLC-immunostaining. *FEBS Lett.* **282** (2), 385-387.

657. Wadstrom,T., Hirmo,S., Novak,H., Guzman,A., Ringner-Pantzar,M., Utt,M., and Aleljung,P. (1997) Sulfatides inhibit binding of Helicobacter pylori to the gastric cancer Kato III cell line. *Curr.Microbiol.* **34** (5), 267-272.

658. Hata,Y., Kita,T., and Murakami,M. (1999) Bovine milk inhibits both adhesion of Helicobacter pylori to sulfatide and Helicobacter pylori-induced vacuolation of vero cells. *Dig.Dis.Sci.* **44** (8), 1696-1702.

659. Chaturvedi,G., Tewari,R., Mrigank, Agnihotri,N., Vishwakarma,R.A., and Ganguly,N.K. (2001) Inhibition of Helicobacter pylori adherence by a peptide derived from neuraminyl lactose binding adhesin. *Mol.Cell Biochem.* **228** (1-2), 83-89.

660. Evans,D.G., Karjalainen,T.K., Evans,D.J., Jr., Graham,D.Y., and Lee,C.H. (1993) Cloning, nucleotide sequence, and expression of a gene encoding an adhesin subunit protein of Helicobacter pylori. *J.Bacteriol.* **175** (3), 674-683.

661. Evans,D.G., Evans,D.J., Jr., Lampert,H.C., and Graham,D.Y. (1995) Restriction fragment length polymorphism in the adhesin gene hpaA of Helicobacter pylori. *Am.J.Gastroenterol.* **90** (8), 1282-1288.

662. Mysore,J.V., Wigginton,T., Simon,P.M., Zopf,D., Heman-Ackah,L.M., and Dubois,A. (1999) Treatment of Helicobacter pylori infection in rhesus monkeys using a novel antiadhesion compound. *Gastroenterology* **117** (6), 1316-1325.

663. O'Toole,P.W., Janzon,L., Doig,P., Huang,J., Kostrzynska,M., and Trust,T.J. (1995) The putative neuraminyllactose-binding hemagglutinin HpaA of Helicobacter pylori CCUG 17874 is a lipoprotein. *J.Bacteriol.* **177** (21), 6049-6057.

664. Osaki,T., Yamaguchi,H., Taguchi,H., Kumada,J., Ogata,S., and Kamiya,S. (1997) Studies on the relationship between adhesive activity and haemagglutination by Helicobacter pylori. *J.Med.Microbiol.* **46** (2), 117-121.

665. Simon,P.M., Goode,P.L., Mobasseri,A., and Zopf,D. (1997) Inhibition of Helicobacter pylori binding to gastrointestinal epithelial cells by sialic acid-containing oligosaccharides. *Infect.Immun.* **65** (2), 750-757.

666. Miller-Podraza,H., Milh,M.A., Teneberg,S., and Karlsson,K.A. (1997) Binding of Helicobacter pylori to sialic acid-containing glycolipids of various origins separated on thin-layer chromatograms. *Infect.Immun.* **65** (6), 2480-2482.

667. Lingwood,C.A., Law,H., Pellizzari,A., Sherman,P., and Drumm,B. (1989) Gastric glycerolipid as a receptor for Campylobacter pylori. *Lancet* **2** (8657), 238-241.

668. Bitzan,M.M., Gold,B.D., Philpott,D.J., Huesca,M., Sherman,P.M., Karch,H., Lissner,R., Lingwood,C.A., and Karmali,M.A. (1998) Inhibition of Helicobacter pylori and Helicobacter mustelae binding to lipid receptors by bovine colostrum. *J.Infect.Dis.* **177** (4), 955-961.

669. Odenbreit,S., Wieland,B., and Haas,R. (1996) Cloning and genetic characterization of Helicobacter pylori catalase and construction of a catalase-deficient mutant strain. *J.Bacteriol.* **178** (23), 6960-6967.

670. Amini,H.R., Ascencio,F., Ruiz-Bustos,E., Romero,M.J., and Wadstrom,T. (1996) Cryptic domains of a 60 kDa heat shock protein of Helicobacter pylori bound to bovine lactoferrin. *FEMS Immunol.Med.Microbiol.* **16** (3-4), 247-255.

671. Huesca,M., Borgia,S., Hoffman,P., and Lingwood,C.A. (1996) Acidic pH changes receptor binding specificity of Helicobacter pylori: a binary adhesion model in which surface heat shock (stress) proteins mediate sulfatide recognition in gastric colonization. *Infect.Immun.* **64** (7), 2643-2648.

672. Huesca,M., Goodwin,A., Bhagwansingh,A., Hoffman,P., and Lingwood,C.A. (1998) Characterization of an acidic-pH-inducible stress protein (hsp70), a putative sulfatide binding adhesin, from Helicobacter pylori. *Infect.Immun.* **66** (9), 4061-4067.

673. Yamaguchi,H., Osaki,T., Taguchi,H., Hanawa,T., Yamamoto,T., and Kamiya,S. (1996) Flow cytometric analysis of the heat shock protein 60 expressed on the cell surface of Helicobacter pylori. *J.Med.Microbiol.* **45** (4), 270-277.

674. Yamaguchi,H., Osaki,T., Kurihara,N., Taguchi,H., Hanawa,T., Yamamoto,T., and Kamiya,S. (1997) Heat-shock protein 60 homologue of Helicobacter pylori is associated with adhesion of H. pylori to human gastric epithelial cells. *J.Med.Microbiol.* **46** (10), 825-831.

675. Yamaguchi,H., Osaki,T., Kurihara,N., Taguchi,H., and Kamiya,S. (1998) [The role of heat shock protein 60 (HSP60) of Helicobacter pylori in adhesion of H. pylori to human gastric epithelial cell]. *Kansenshogaku Zasshi* **72** (5), 487-492.

676. de Jonge,R., Durrani,Z., Rijpkema,S.G., Kuipers,E.J., van Vliet,A.H., and Kusters,J.G. (2004) Role of the Helicobacter pylori outer-membrane proteins AlpA and AlpB in colonization of the guinea pig stomach. *J.Med.Microbiol.* **53** (Pt 5), 375-379.

677. Odenbreit,S., Till,M., Hofreuter,D., Faller,G., and Haas,R. (1999) Genetic and functional characterization of the alpAB gene locus essential for the adhesion of Helicobacter pylori to human gastric tissue. *Mol.Microbiol.* **31** (5), 1537-1548.

678. Yamaoka,Y., Kikuchi,S., El Zimaity,H.M., Gutierrez,O., Osato,M.S., and Graham,D.Y. (2002) Importance of Helicobacter pylori oipA in clinical presentation, gastric inflammation, and mucosal interleukin 8 production. *Gastroenterology* **123** (2), 414-424.

679. de Jonge,R., Pot,R.G., Loffeld,R.J., van Vliet,A.H., Kuipers,E.J., and Kusters,J.G. (2004) The functional status of the Helicobacter pylori sabB adhesin gene as a putative marker for disease outcome. *Helicobacter.* **9** (2), 158-164.

680. Odenbreit,S., Kavermann,H., Puls,J., and Haas,R. (2002) CagA tyrosine phosphorylation and interleukin-8 induction by Helicobacter pylori are independent from alpAB, HopZ and bab group outer membrane proteins. *Int.J.Med.Microbiol.* **292** (3-4), 257-266.

681. Peck,B., Ortkamp,M., Diehl,K.D., Hundt,E., and Knapp,B. (1999) Conservation, localization and expression of HopZ, a protein involved in adhesion of Helicobacter pylori. *Nucleic Acids Res.* **27** (16), 3325-3333.

682. Yamaoka,Y., Kita,M., Kodama,T., Imamura,S., Ohno,T., Sawai,N., Ishimaru,A., Imanishi,J., and Graham,D.Y. (2002) Helicobacter pylori infection in mice: Role of outer membrane proteins in colonization and inflammation. *Gastroenterology* **123** (6), 1992-2004.

683. Rubinsztein-Dunlop,S., Guy,B., Lissolo,L., and Fischer,H. (2005) Identification of two new Helicobacter pylori surface proteins involved in attachment to epithelial cell lines. *J.Med.Microbiol.* **54** (Pt 5), 427-434.

684. Collins,J.S. (1992) Role of Helicobacter pylori in gastritis and duodenitis in man. *Agents Actions* **Spec No** C47-C49.

685. Kaji,T., Ishihara,S., Ashizawa,N., Hamamoto,N., Endo,H., Fukuda,R., Adachi,K., Watanabe,M., Nakao,M., and Kinoshita,Y. (2002) Adherence of Helicobacter pylori to gastric epithelial cells and mucosal inflammation. *J Lab Clin.Med.* **139** (4), 244-250.

686. Suzuki,H., Masaoka,T., Miyazawa,M., Suzuki,M., Miura,S., and Ishii,H. (2002) Gastric mucosal response to Helicobacter pylori. *Keio J Med.* **51 Suppl 2** 40-44.

687. Kim,S.Y., Lee,Y.C., Kim,H.K., and Blaser,M.J. (2006) Helicobacter pylori CagA transfection of gastric epithelial cells induces interleukin-8. *Cell Microbiol* **8** (1), 97-106.

688. Yamaoka,Y., Kudo,T., Lu,H., Casola,A., Brasier,A.R., and Graham,D.Y. (2004) Role of interferon-stimulated responsive element-like element in interleukin-8 promoter in Helicobacter pylori infection. *Gastroenterology* **126** (4), 1030-1043.

689. Bliss,C.M., Jr., Golenbock,D.T., Keates,S., Linevsky,J.K., and Kelly,C.P. (1998) Helicobacter pylori lipopolysaccharide binds to CD14 and stimulates release of interleukin-8, epithelial neutrophil-activating peptide 78, and monocyte chemotactic protein 1 by human monocytes. *Infect Immun.* **66** (11), 5357-5363.

690. Hansen,P.S., Petersen,S.B., Varning,K., and Nielsen,H. (2002) Additive effects of Helicobacter pylori lipopolysaccharide and proteins in monocyte inflammatory responses. *Scand.J Gastroenterol.* **37** (7), 765-771.

691. Harris,P.R., Ernst,P.B., Kawabata,S., Kiyono,H., Graham,M.F., and Smith,P.D. (1998) Recombinant Helicobacter pylori urease activates primary mucosal macrophages. *J Infect Dis* **178** (5), 1516-1520.

692. Mohammadi,M., Czinn,S., Redline,R., and Nedrud,J. (1996) Helicobacter-specific cell-mediated immune responses display a predominant Th1 phenotype and promote a delayed-type hypersensitivity response in the stomachs of mice. *J Immunol.* **156** (12), 4729-4738.

693. Bodger,K. and Crabtree,J.E. (1998) Helicobacter pylori and gastric inflammation. *Br.Med.Bull.* **54** (1), 139-150.

694. Bergman,M.P., Engering,A., Smits,H.H., van Vliet,S.J., van Bodegraven,A.A., Wirth,H.P., Kapsenberg,M.L., Vandenbroucke-Grauls,C.M., van Kooyk,Y., and Appelmelk,B.J. (2004) Helicobacter pylori modulates the T helper cell 1/T helper cell 2 balance through phase-variable interaction between lipopolysaccharide and DC-SIGN. *J.Exp.Med.* **200** (8), 979-990.

695. Mohammadi,M., Nedrud,J., Redline,R., Lycke,N., and Czinn,S.J. (1997) Murine CD4 T-cell response to Helicobacter infection: TH1 cells enhance gastritis and TH2 cells reduce bacterial load. *Gastroenterology* **113** (6), 1848-1857.

696. Hoffman,P.S., Vats,N., Hutchison,D., Butler,J., Chisholm,K., Sisson,G., Raudonikiene,A., Marshall,J.S., and Veldhuyzen van Zanten,S.J. (2003) Development of an interleukin-12-deficient mouse model that is permissive for colonization by a motile KE26695 strain of Helicobacter pylori. *Infect.Immun.* **71** (5), 2534-2541.

697. Ceponis,P.J., McKay,D.M., Menaker,R.J., Galindo-Mata,E., and Jones,N.L. (2003) Helicobacter pylori infection interferes with epithelial Stat6-mediated interleukin-4 signal transduction independent of cagA, cagE, or VacA. *J.Immunol.* **171** (4), 2035-2041.

698. Holck,S., Norgaard,A., Bennedsen,M., Permin,H., Norn,S., and Andersen,L.P. (2003) Gastric mucosal cytokine responses in Helicobacter pylori-infected patients with gastritis and peptic ulcers. Association with inflammatory parameters and bacteria load. *FEMS Immunol.Med.Microbiol.* **36** (3), 175-180.

699. Zavros,Y. and Merchant,J.L. (2005) Modulating the cytokine response to treat Helicobacter gastritis. *Biochem.Pharmacol.* **69** (3), 365-371.

700. Zavros,Y., Rathinavelu,S., Kao,J.Y., Todisco,A., Del Valle,J., Weinstock,J.V., Low,M.J., and Merchant,J.L. (2003) Treatment of Helicobacter gastritis with IL-4 requires somatostatin. *Proc.Natl.Acad.Sci.U.S.A* **100** (22), 12944-12949.

701. Smith,M.F., Jr., Mitchell,A., Li,G., Ding,S., Fitzmaurice,A.M., Ryan,K., Crowe,S., and Goldberg,J.B. (2003) Toll-like receptor (TLR) 2 and TLR5, but not TLR4, are required for Helicobacter pylori-induced NF-kappa B activation and chemokine expression by epithelial cells. *J.Biol.Chem.* **278** (35), 32552-32560.

702. Su,B., Ceponis,P.J., Lebel,S., Huynh,H., and Sherman,P.M. (2003) Helicobacter pylori activates Toll-like receptor 4 expression in gastrointestinal epithelial cells. *Infect.Immun.* **71** (6), 3496-3502.

703. Lepper,P.M., Triantafilou,M., Schumann,C., Schneider,E.M., and Triantafilou,K. (2005) Lipopolysaccharides from Helicobacter pylori can act as antagonists for Toll-like receptor 4. *Cell Microbiol.* **7** (4), 519-528.

704. Rad,R., Gerhard,M., Lang,R., Schoniger,M., Rosch,T., Schepp,W., Becker,I., Wagner,H., and Prinz,C. (2002) The Helicobacter pylori blood group antigen-binding adhesin facilitates bacterial colonization and augments a nonspecific immune response. *J.Immunol.* **168** (6), 3033-3041.

705. Crabtree,J.E., Covacci,A., Farmery,S.M., Xiang,Z., Tompkins,D.S., Perry,S., Lindley,I.J., and Rappuoli,R. (1995) Helicobacter pylori induced interleukin-8 expression in gastric epithelial cells is associated with CagA positive phenotype. *J.Clin.Pathol.* **48** (1), 41-45.

706. Crabtree,J.E. (1996) Immune and inflammatory responses to Helicobacter pylori infection. *Scand.J.Gastroenterol.Suppl* **215** 3-10.

707. Basu,M., Czinn,S.J., and Blanchard,T.G. (2004) Absence of catalase reduces long-term survival of Helicobacter pylori in macrophage phagosomes. *Helicobacter.* **9** (3), 211-216.

708. Kwok,T., Backert,S., Schwarz,H., Berger,J., and Meyer,T.F. (2002) Specific entry of Helicobacter pylori into cultured gastric epithelial cells via a zipper-like mechanism. *Infect.Immun.* **70** (4), 2108-2120.

709. Amieva,M.R., Salama,N.R., Tompkins,L.S., and Falkow,S. (2002) Helicobacter pylori enter and survive within multivesicular vacuoles of epithelial cells. *Cell Microbiol.* **4** (10), 677-690.

710. Harris,P.R., Mobley,H.L., Perez-Perez,G.I., Blaser,M.J., and Smith,P.D. (1996) Helicobacter pylori urease is a potent stimulus of mononuclear phagocyte activation and inflammatory cytokine production. *Gastroenterology* **111** (2), 419-425.

711. Ferrero,R.L., Thiberge,J.M., Huerre,M., and Labigne,A. (1994) Recombinant antigens prepared from the urease subunits of Helicobacter spp.: evidence of protection in a mouse model of gastric infection. *Infect.Immun.* **62** (11), 4981-4989.

712. Hynes,S.O., Keenan,J.I., Ferris,J.A., Annuk,H., and Moran,A.P. (2005) Lewis epitopes on outer membrane vesicles of relevance to Helicobacter pylori pathogenesis. *Helicobacter.* **10** (2), 146-156.

713. Ismail,S., Hampton,M.B., and Keenan,J.I. (2003) Helicobacter pylori outer membrane vesicles modulate proliferation and interleukin-8 production by gastric epithelial cells. *Infect Immun.* **71** (10), 5670-5675.

714. Crabtree,J.E., Shallcross,T.M., Wyatt,J.I., Taylor,J.D., Heatley,R.V., Rathbone,B.J., and Losowsky,M.S. (1991) Mucosal humoral immune response to Helicobacter pylori in patients with duodenitis. *Dig.Dis.Sci.* **36** (9), 1266-1273.

715. Crabtree,J.E., Taylor,J.D., Wyatt,J.I., Heatley,R.V., Shallcross,T.M., Tompkins,D.S., and Rathbone,B.J. (1991) Mucosal IgA recognition of Helicobacter pylori 120 kDa protein, peptic ulceration, and gastric pathology. *Lancet* **338** (8763), 332-335.

716. Wyatt,J.I., Rathbone,B.J., and Heatley,R.V. (1986) Local immune response to gastric Campylobacter in non-ulcer dyspepsia. *J Clin.Pathol.* **39** (8), 863-870.

717. Mattsson,A., Quiding-Jarbrink,M., Lonroth,H., Hamlet,A., Ahlstedt,I., and Svennerholm,A. (1998) Antibody-secreting cells in the stomachs of symptomatic and asymptomatic Helicobacter pylori-infected subjects. *Infect.Immun.* **66** (6), 2705-2712.

718. Tinnert,A., Mattsson,A., Bolin,I., Dalenback,J., Hamlet,A., and Svennerholm,A.M. (1997) Local and systemic immune responses in humans against Helicobacter pylori antigens from homologous and heterologous strains. *Microb.Pathog.* **23** (5), 285-296.

719. Tosi,M.F. and Czinn,S.J. (1990) Opsonic activity of specific human IgG against Helicobacter pylori. *J Infect Dis* **162** (1), 156-162.

720. Kranzer,K., Eckhardt,A., Aigner,M., Knoll,G., Deml,L., Speth,C., Lehn,N., Rehli,M., and Schneider-Brachert,W. (2004) Induction of maturation and cytokine release of human dendritic cells by Helicobacter pylori. *Infect.Immun.* **72** (8), 4416-4423.

721. Mattsson,A., Tinnert,A., Hamlet,A., Lonroth,H., Bolin,I., and Svennerholm,A.M. (1998) Specific antibodies in sera and gastric aspirates of symptomatic and asymptomatic Helicobacter pylori-infected subjects. *Clin.Diagn.Lab Immunol.* **5** (3), 288-293.

722. Futagami,S., Takahashi,H., Norose,Y., and Kobayashi,M. (1998) Systemic and local immune responses against Helicobacter pylori urease in patients with chronic gastritis: distinct IgA and IgG productive sites. *Gut* **43** (2), 168-175.

723. Krauss-Etschmann,S., Gruber,R., Plikat,K., Antoni,I., Demmelmair,H., Reinhardt,D., and Koletzko,S. (2005) Increase of antigen-presenting cells in the gastric mucosa of Helicobacter pylori-infected children. *Helicobacter.* **10** (3), 214-222.

724. Gobert,A.P., Bambou,J.C., Werts,C., Balloy,V., Chignard,M., Moran,A.P., and Ferrero,R.L. (2004) Helicobacter pylori heat shock protein 60 mediates interleukin-6 production by macrophages via a toll-like receptor (TLR)-2-, TLR-4-, and myeloid differentiation factor 88-independent mechanism. *J Biol.Chem.* **279** (1), 245-250.

725. Nurgalieva,Z.Z., Conner,M.E., Opekun,A.R., Zheng,C.Q., Elliott,S.N., Ernst,P.B., Osato,M., Estes,M.K., and Graham,D.Y. (2005) B-cell and T-cell immune responses to experimental Helicobacter pylori infection in humans. *Infect Immun.* **73** (5), 2999-3006.

726. Allen,L.A. (2000) Modulating phagocyte activation: the pros and cons of Helicobacter pylori virulence factors. *J.Exp.Med.* **191** (9), 1451-1454.

727. Allen,L.A., Schlesinger,L.S., and Kang,B. (2000) Virulent strains of Helicobacter pylori demonstrate delayed phagocytosis and stimulate homotypic phagosome fusion in macrophages. *J.Exp.Med.* **191** (1), 115-128.

728. Allen,L.A., Beecher,B.R., Lynch,J.T., Rohner,O.V., and Wittine,L.M. (2005) Helicobacter pylori disrupts NADPH oxidase targeting in human neutrophils to induce extracellular superoxide release. *J Immunol.* **174** (6), 3658-3667.

729. McCord,J.M. and Fridovich,I. (1969) The utility of superoxide dismutase in studying free radical reactions. I. Radicals generated by the interaction of sulfite, dimethyl sulfoxide, and oxygen. *J.Biol.Chem.* **244** (22), 6056-6063.

730. Backhed,F., Rokbi,B., Torstensson,E., Zhao,Y., Nilsson,C., Seguin,D., Normark,S., Buchan,A.M., and Richter-Dahlfors,A. (2003) Gastric mucosal recognition of Helicobacter pylori is independent of Toll-like receptor 4. *J Infect Dis* **187** (5), 829-836.

731. Dalpke,A., Zimmermann,S., and Heeg,K. (2002) CpG DNA in the prevention and treatment of infections. *BioDrugs.* **16** (6), 419-431.

732. Ando,T., Wassenaar,T.M., Peek,R.M., Jr., Aras,R.A., Tschumi,A.I., van Doorn,L.J., Kusugami,K., and Blaser,M.J. (2002) A Helicobacter pylori restriction endonuclease-replacing gene, hrgA, is associated with gastric cancer in Asian strains. *Cancer Res.* **62** (8), 2385-2389.

733. Xu,Q., Morgan,R.D., Roberts,R.J., and Blaser,M.J. (2000) Identification of type II restriction and modification systems in Helicobacter pylori reveals their substantial diversity among strains. *Proc.Natl.Acad.Sci.U.S.A* **97** (17), 9671-9676.

734. Boncristiano,M., Paccani,S.R., Barone,S., Ulivieri,C., Patrussi,L., Ilver,D., Amedei,A., D'Elios,M.M., Telford,J.L., and Baldari,C.T. (2003) The Helicobacter pylori vacuolating toxin inhibits T cell activation by two independent mechanisms. *J.Exp.Med.* **198** (12), 1887-1897.

735. Sundrud,M.S., Torres,V.J., Unutmaz,D., and Cover,T.L. (2004) Inhibition of primary human T cell proliferation by Helicobacter pylori vacuolating toxin (VacA) is independent of VacA effects on IL-2 secretion. *Proc.Natl.Acad.Sci.U.S.A* **101** (20), 7727-7732.

736. Wang,J., Brooks,E.G., Bamford,K.B., Denning,T.L., Pappo,J., and Ernst,P.B. (2001) Negative selection of T cells by Helicobacter pylori as a model for bacterial strain selection by immune evasion. *J Immunol.* **167** (2), 926-934.

737. Lundgren,A., Suri-Payer,E., Enarsson,K., Svennerholm,A.M., and Lundin,B.S. (2003) Helicobacter pylori-specific CD4+ CD25high regulatory T cells suppress memory T-cell responses to H. pylori in infected individuals. *Infect.Immun.* **71** (4), 1755-1762.

738. Aras,R.A., Fischer,W., Perez-Perez,G.I., Crosatti,M., Ando,T., Haas,R., and Blaser,M.J. (2003) Plasticity of repetitive DNA sequences within a bacterial (Type IV) secretion system component. *J Exp.Med.* **198** (9), 1349-1360.

739. Pride,D.T., Meinersmann,R.J., and Blaser,M.J. (2001) Allelic Variation within Helicobacter pylori babA and babB. *Infect Immun.* **69** (2), 1160-1171.

740. Tomb,J.F., White,O., Kerlavage,A.R., Clayton,R.A., Sutton,G.G., Fleischmann,R.D., Ketchum,K.A., Klenk,H.P., Gill,S., Dougherty,B.A., Nelson,K., Quackenbush,J., Zhou,L., Kirkness,E.F., Peterson,S., Loftus,B., Richardson,D., Dodson,R., Khalak,H.G., Glodek,A., McKenney,K., Fitzegerald,L.M., Lee,N., Adams,M.D., Hickey,E.K., Berg,D.E., Gocayne,J.D., Utterback,T.R., Peterson,J.D., Kelley,J.M., Cotton,M.D., Weidman,J.M., Fujii,C., Bowman,C., Watthey,L., Wallin,E., Hayes,W.S., Borodovsky,M., Karp,P.D., Smith,H.O., Fraser,C.M., and Venter,J.C. (1997) The complete genome sequence of the gastric pathogen Helicobacter pylori. *Nature* **388** (6642), 539-547.

741. Owen,M.K. and Clenney,T.L. (2004) Management of vaginitis. *Am.Fam.Physician* **70** (11), 2125-2132.

742. Sobel,J.D., Faro,S., Force,R.W., Foxman,B., Ledger,W.J., Nyirjesy,P.R., Reed,B.D., and Summers,P.R. (1998) Vulvovaginal candidiasis: epidemiologic, diagnostic, and therapeutic considerations. *Am.J Obstet.Gynecol.* **178** (2), 203-211.

743. Sobel,J.D. (1997) Vaginitis. *N.Engl.J Med.* **337** (26), 1896-1903.

744. Sobel,J.D., Kapernick,P.S., Zervos,M., Reed,B.D., Hooton,T., Soper,D., Nyirjesy,P., Heine,M.W., Willems,J., Panzer,H., and Wittes,H. (2001) Treatment of complicated Candida vaginitis: comparison of single and sequential doses of fluconazole. *Am.J.Obstet.Gynecol.* **185** (2), 363-369.

745. Haefner,H.K. (1999) Current evaluation and management of vulvovaginitis. *Clin.Obstet.Gynecol.* **42** (2), 184-195.

746. Sobel,J.D. (2002) Vulvovaginal candidiasis: a comparison of HIV-positive and -negative women. *Int.J STD AIDS* **13** (6), 358-362.

747. McLoughlin,R., Racz,I., Buckley,M., O'Connor,H.J., and O'Morain,C. (2004) Therapy of Helicobacter pylori. *Helicobacter.* **9 Suppl 1** 42-48.

748. Malfertheiner,P., Megraud,F., O'Morain,C., Hungin,A.P., Jones,R., Axon,A., Graham,D.Y., and Tytgat,G. (2002) Current concepts in the management of Helicobacter pylori infection--the Maastricht 2-2000 Consensus Report. *Aliment.Pharmacol.Ther.* **16** (2), 167-180.

749. Delaney,B., Moayyedi,P., and Forman,D. (2002) Helicobacter pylori infection. *Clin.Evid.* (8), 453-468.

750. Zullo,A., Hassan,C., Lorenzetti,R., and Morini,S. (2001) Helicobacter pylori eradication: do we have another ace up our sleeve? *Dig.Liver Dis.* **33** (9), 805-806.

751. Gomollon,F., Ducons,J.A., Ferrero,M., Garcia,C.J., Guirao,R., Simon,M.A., and Montoro,M. (1999) Quadruple therapy is effective for eradicating Helicobacter pylori after failure of triple proton-pump inhibitor-based therapy: a detailed, prospective analysis of 21 consecutive cases. *Helicobacter.* **4** (4), 222-225.

752. Playford,R.J., Podas,T., and Modlin,I. (1999) Pantoprazole, Prout and the proton pump. *Hosp.Med.* **60** (7), 500-504.

753. Welage,L.S. and Berardi,R.R. (2000) Evaluation of omeprazole, lansoprazole, pantoprazole, and rabeprazole in the treatment of acid-related diseases. *J.Am.Pharm.Assoc.(Wash.)* **40** (1), 52-62.

754. Williams,M.P. and Pounder,R.E. (1999) Review article: the pharmacology of rabeprazole. *Aliment.Pharmacol.Ther.* **13 Suppl 3** 3-10.

755. Schubert,M.L. and Shamburek,R.D. (1990) Control of acid secretion. *Gastroenterol.Clin.North Am.* **19** (1), 1-25.

756. Tytgat,G.N. and Nio,C.Y. (1987) Mucosa protectives: sucralfate and colloidal bismuth subcitrate in peptic ulcer disease. *Z.Gastroenterol.* **25 Suppl 3** 152-161.

757. McNulty,C.A., Dent,J., and Wise,R. (1985) Susceptibility of clinical isolates of Campylobacter pyloridis to 11 antimicrobial agents. *Antimicrob.Agents Chemother.* **28** (6), 837-838.

758. McNulty,C.A. (1990) Bismuth subsalicylate in the treatment of gastritis due to Campylobacter pylori. *Rev.Infect Dis* **12 Suppl 1** S94-S98.

759. Rauws,E.A., Langenberg,W., Houthoff,H.J., Zanen,H.C., and Tytgat,G.N. (1988) Campylobacter pyloridis-associated chronic active antral gastritis. A prospective study of its prevalence and the effects of antibacterial and antiulcer treatment. *Gastroenterology* **94** (1), 33-40.

760. Coghlan,J.G., Gilligan,D., Humphries,H., McKenna,D., Dooley,C., Sweeney,E., Keane,C., and O'Morain,C. (1987) Campylobacter pylori and recurrence of duodenal ulcers--a 12-month follow-up study. *Lancet* **2** (8568), 1109-1111.

761. Marshall,B.J., Armstrong,J.A., Francis,G.J., Nokes,N.T., and Wee,S.H. (1987) Antibacterial action of bismuth in relation to Campylobacter pyloridis colonization and gastritis. *Digestion* **37 Suppl 2** 16-30.

762. Gisbert,J.P., Khorrami,S., Calvet,X., Gabriel,R., Carballo,F., and Pajares,J.M. (2003) Meta-analysis: proton pump inhibitors vs. H2-receptor antagonists--their efficacy with antibiotics in Helicobacter pylori eradication. *Aliment.Pharmacol.Ther.* **18** (8), 757-766.

763. Vergara,M., Vallve,M., Gisbert,J.P., and Calvet,X. (2003) Meta-analysis: comparative efficacy of different proton-pump inhibitors in triple therapy for Helicobacter pylori eradication. *Aliment.Pharmacol.Ther.* **18** (6), 647-654.

764. Dore,M.P., Graham,D.Y., Mele,R., Marras,L., Nieddu,S., Manca,A., and Realdi,G. (2002) Colloidal bismuth subcitrate-based twice-a-day quadruple therapy as primary or salvage therapy for Helicobacter pylori infection. *Am.J.Gastroenterol.* **97** (4), 857-860.

765. Gisbert,J.P., Calvet,X., Bujanda,L., Marcos,S., Gisbert,J.L., and Pajares,J.M. (2003) 'Rescue' therapy with rifabutin after multiple Helicobacter pylori treatment failures. *Helicobacter.* **8** (2), 90-94.

766. Perri,F., Qasim,A., Marras,L., and O'Morain,C. (2003) Treatment of Helicobacter pylori infection. *Helicobacter.* **8 Suppl 1** 53-60.

767. Qasim,A., Sebastian,S., Thornton,O., Dobson,M., McLoughlin,R., Buckley,M., O'Connor,H., and O'Morain,C. (2005) Rifabutin- and furazolidone-based Helicobacter pylori eradication therapies after failure of standard first- and second-line eradication attempts in dyspepsia patients. *Aliment.Pharmacol.Ther.* **21** (1), 91-96.

768. Di Caro,S., Ojetti,V., Zocco,M.A., Cremonini,F., Bartolozzi,F., Candelli,M., Lupascu,A., Nista,E.C., Cammarota,G., and Gasbarrini,A. (2002) Mono, dual and triple moxifloxacin-based therapies for Helicobacter pylori eradication. *Aliment.Pharmacol.Ther.* **16** (3), 527-532.

769. Cammarota,G., Cianci,R., Cannizzaro,O., Cuoco,L., Pirozzi,G., Gasbarrini,A., Armuzzi,A., Zocco,M.A., Santarelli,L., Arancio,F., and Gasbarrini,G. (2000) Efficacy of two one-week rabeprazole/levofloxacin-based triple therapies for Helicobacter pylori infection. *Aliment.Pharmacol.Ther.* **14** (10), 1339-1343.

770. Watanabe,Y., Aoyama,N., Shirasaka,D., Maekawa,S., Kuroda,K., Miki,I., Kachi,M., Fukuda,M., Wambura,C., Tamura,T., and Kasuga,M. (2003) Levofloxacin based triple therapy as a second-line treatment after failure of helicobacter pylori eradication with standard triple therapy. *Dig.Liver Dis.* **35** (10), 711-715.

771. Zullo,A., Hassan,C., De,F., V, Lorenzetti,R., Marignani,M., Angeletti,S., Ierardi,E., and Morini,S. (2003) A third-line levofloxacin-based rescue therapy for Helicobacter pylori eradication. *Dig.Liver Dis.* **35** (4), 232-236.

772. McLoughlin,R.M., O'Morain,C.A., and O'Connor,H.J. (2005) Eradication of Helicobacter pylori: recent advances in treatment. *Fundam.Clin.Pharmacol.* **19** (4), 421-427.

773. Ojetti,V., Migneco,A., Zocco,M.A., Nista,E.C., Gasbarrini,G., and Gasbarrini,A. (2004) Beta-lactamase inhibitor enhances Helicobacter pylori eradication rate. *J.Intern.Med.* **255** (1), 125-129.

774. Roghani,H.S., Massarrat,S., Shirekhoda,M., and Butorab,Z. (2003) Effect of different doses of furazolidone with amoxicillin and omeprazole on eradication of Helicobacter pylori. *J.Gastroenterol.Hepatol.* **18** (7), 778-782.

775. Frota,L.C., da Cunha,M.P., Luz,C.R., Araujo-Filho,A.H., Frota,L.A., and Braga,L.L. (2005) Helicobacter pylori eradication using tetracycline and furazolidone versus amoxicillin and azithromycin in lansoprazole based triple therapy: an open randomized clinical trial. *Arq Gastroenterol.* **42** (2), 111-115.

776. Wang,F., Wang,X.Y., Jia,Y., Shen,S.R., Xu,C.X., Tang,W.L., Liu,S.J., and Chen,X. (2004) [Five-day rabeprazole-based triple therapy for helicobacter pylori infection]. *Zhong.Nan.Da.Xue.Xue.Bao.Yi.Xue.Ban.* **29** (5), 589-591.

777. Dai,G., Cheng,N., Dong,L., Muramatsu,M., Xiao,S., Wang,M.W., and Zhu,D.X. (2005) Bactericidal and morphological effects of NE-2001, a novel synthetic agent directed against Helicobacter pylori. *Antimicrob.Agents Chemother.* **49** (8), 3468-3473.

778. Araujo,C.R., Pinto Valdivia,J.L., Ramirez,D., Cok,G.J., and Bussalleu,R.A. (2005) [New ultrashort scheme for helicobacter pylori infection eradication using tetracyline, furazolidone and colloidal bismuth subcitrate in dyspeptic patients with or without peptic ulceration in the National Hospital Cayetano Heredia]. *Rev.Gastroenterol.Peru* **25** (1), 23-41.

779. Zullo,A., Vaira,D., Vakil,N., Hassan,C., Gatta,L., Ricci,C., De,F., V, Menegatti,M., Tampieri,A., Perna,F., Rinaldi,V., Perri,F., Papadia,C., Fornari,F., Pilati,S., Mete,L.S., Merla,A., Poti,R., Marinone,G., Savioli,A., Campo,S.M., Faleo,D., Ierardi,E., Miglioli,M., and Morini,S. (2003) High eradication rates of Helicobacter pylori with a new sequential treatment. *Aliment.Pharmacol.Ther.* **17** (5), 719-726.

780. Hassan,C., De,F., V, Zullo,A., Scaccianoce,G., Piglionica,D., Ierardi,E., Panella,C., and Morini,S. (2003) Sequential treatment for Helicobacter pylori eradication in duodenal ulcer patients: improving the cost of pharmacotherapy. *Aliment.Pharmacol.Ther.* **18** (6), 641-646.

781. De,F., V, Della,V.N., Stoppino,V., Amoruso,A., Muscatiello,N., Panella,C., and Ierardi,E. (2004) Effectiveness and pharmaceutical cost of sequential treatment for Helicobacter pylori in patients with non-ulcer dyspepsia. *Aliment.Pharmacol.Ther.* **19** (9), 993-998.

782. De,F., V, Zullo,A., Margiotta,M., Marangi,S., Burattini,O., Berloco,P., Russo,F., Barone,M., Di Leo,A., Minenna,M.F., Stoppino,V., Morini,S., Panella,C., Francavilla,A., and Ierardi,E. (2004) Sequential treatment for Helicobacter pylori does not share the risk factors of triple therapy failure. *Aliment.Pharmacol.Ther.* **19** (4), 407-414.

783. Hojo,M., Miwa,H., Nagahara,A., and Sato,N. (2001) Pooled analysis on the efficacy of the second-line treatment regimens for Helicobacter pylori infection. *Scand.J.Gastroenterol.* **36** (7), 690-700.

784. Sanders,C.C. and Sanders,W.E. (1995) Resistance to antibacterial agents. In: Jungkind,D.I., Mortensen,J.E., Fraimow,H.S., and Calandra,G.B. (eds), Plenum Press, New York, NY. USA.

785. Prasad,R. and Kapoor,K. (2005) Multidrug resistance in yeast Candida. *Int.Rev.Cytol.* **242** 215-248.

786. Lamb,D.C., Kelly,D.E., Manning,N.J., and Kelly,S.L. (1997) Reduced intracellular accumulation of azole antifungal results in resistance in Candida albicans isolate NCPF 3363. *FEMS Microbiol.Lett.* **147** (2), 189-193.

787. Pasrija,R., Krishnamurthy,S., Prasad,T., Ernst,J.F., and Prasad,R. (2005) Squalene epoxidase encoded by ERG1 affects morphogenesis and drug susceptibilities of Candida albicans. *J Antimicrob.Chemother.* **55** (6), 905-913.

788. Haynes,M.P., Chong,P.L., Buckley,H.R., and Pieringer,R.A. (1996) Fluorescence studies on the molecular action of amphotericin B on susceptible and resistant fungal cells. *Biochemistry* **35** (24), 7983-7992.

789. Denning,D.W. (2003) Echinocandin antifungal drugs. *Lancet* **362** (9390), 1142-1151.

790. Diasio,R.B., Bennett,J.E., and Myers,C.E. (1978) Mode of action of 5-fluorocytosine. *Biochem.Pharmacol.* **27** (5), 703-707.

791. Ghannoum,M.A. and Rice,L.B. (1999) Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clin.Microbiol Rev.* **12** (4), 501-517.

792. Balkis,M.M., Leidich,S.D., Mukherjee,P.K., and Ghannoum,M.A. (2002) Mechanisms of fungal resistance: an overview. *Drugs* **62** (7), 1025-1040.

793. Morschhauser,J. (2002) The genetic basis of fluconazole resistance development in Candida albicans. *Biochim.Biophys.Acta* **1587** (2-3), 240-248.

794. Marichal,P., Koymans,L., Willemsens,S., Bellens,D., Verhasselt,P., Luyten,W., Borgers,M., Ramaekers,F.C., Odds,F.C., and Bossche,H.V. (1999) Contribution of mutations in the cytochrome P450 14alpha-demethylase (Erg11p, Cyp51p) to azole resistance in Candida albicans. *Microbiology* **145 (Pt 10)** 2701-2713.

795. Favre,B., Didmon,M., and Ryder,N.S. (1999) Multiple amino acid substitutions in lanosterol 14alpha-demethylase contribute to azole resistance in Candida albicans. *Microbiology* **145 (Pt 10)** 2715-2725.

796. Kakeya,H., Miyazaki,Y., Miyazaki,H., Nyswaner,K., Grimberg,B., and Bennett,J.E. (2000) Genetic analysis of azole resistance in the Darlington strain of Candida albicans. *Antimicrob.Agents Chemother.* **44** (11), 2985-2990.

797. Kelly,S.L., Lamb,D.C., Loeffler,J., Einsele,H., and Kelly,D.E. (1999) The G464S amino acid substitution in Candida albicans sterol 14alpha-demethylase causes fluconazole resistance in the clinic through reduced affinity. *Biochem.Biophys.Res.Commun.* **262** (1), 174-179.

798. Lamb,D.C., Kelly,D.E., Schunck,W.H., Shyadehi,A.Z., Akhtar,M., Lowe,D.J., Baldwin,B.C., and Kelly,S.L. (1997) The mutation T315A in Candida albicans sterol 14alpha-demethylase causes reduced enzyme activity and fluconazole resistance through reduced affinity. *J Biol.Chem.* **272** (9), 5682-5688.

799. Loffler,J., Kelly,S.L., Hebart,H., Schumacher,U., Lass-Florl,C., and Einsele,H. (1997) Molecular analysis of cyp51 from fluconazole-resistant Candida albicans strains. *FEMS Microbiol Lett.* **151** (2), 263-268.

800. Prasad,R., Murthy,S.K., Gupta,V., and Prasad,R. (1995) Multiple drug resistance in Candida albicans. *Acta Biochim.Pol.* **42** (4), 497-504.

801. Sanglard,D., Kuchler,K., Ischer,F., Pagani,J.L., Monod,M., and Bille,J. (1995) Mechanisms of resistance to azole antifungal agents in Candida albicans isolates from AIDS patients involve specific multidrug transporters. *Antimicrob.Agents Chemother.* **39** (11), 2378-2386.

802. Loffler,J., Einsele,H., Hebart,H., Schumacher,U., Hrastnik,C., and Daum,G. (2000) Phospholipid and sterol analysis of plasma membranes of azole-resistant Candida albicans strains. *FEMS Microbiol.Lett.* **185** (1), 59-63.

803. Mukhopadhyay,K., Kohli,A., and Prasad,R. (2002) Drug susceptibilities of yeast cells are affected by membrane lipid composition. *Antimicrob.Agents Chemother.* **46** (12), 3695-3705.

804. Cohen,L.E., Anderson,J.B., and Kohn,L.M. (2002) Evolution of drug resistance in *Candida albicans*. *Ann Rev Microbiol* **56** 139-165.
Ref ID: 2376

805. Odds,F. (2004) The evolution of antifungal resistance in *Candida* species. *Microbiology Today* **31** 166-167.

806. Hua,J.S., Zheng,P.Y., Fong,T.K., Khin,M.M., and Bow,H. (1998) Helicobacter pylori acquistion of metronidazole resistance by natural transformation in vitro. *World J.Gastroenterol.* **4** (5), 385-387.

807. Megraud,F. (2001) Resistance of Helicobacter pylori to antibiotics and its impact on treatment options. *Drug Resist.Updat.* **4** (3), 178-186.

808. Stone,G.G., Shortridge,D., Flamm,R.K., Versalovic,J., Beyer,J., Idler,K., Zulawinski,L., and Tanaka,S.K. (1996) Identification of a 23S rRNA gene mutation in clarithromycin-resistant Helicobacter pylori. *Helicobacter.* **1** (4), 227-228.

809. Taylor,D.E., Ge,Z., Purych,D., Lo,T., and Hiratsuka,K. (1997) Cloning and sequence analysis of two copies of a 23S rRNA gene from Helicobacter pylori and association of clarithromycin resistance with 23S rRNA mutations. *Antimicrob.Agents Chemother.* **41** (12), 2621-2628.

810. Occhialini,A., Urdaci,M., Doucet-Populaire,F., Bebear,C.M., Lamouliatte,H., and Megraud,F. (1997) Macrolide resistance in Helicobacter pylori: rapid detection of point mutations and assays of macrolide binding to ribosomes. *Antimicrob.Agents Chemother.* **41** (12), 2724-2728.

811. Megraud,F. (2003) Helicobacter pylori resistance to antibiotics: prevalence, mechanism, detection. What's new? *Can.J.Gastroenterol.* **17 Suppl B** 49B-52B.

812. Megraud,F. (2004) H pylori antibiotic resistance: prevalence, importance, and advances in testing. *Gut* **53** (9), 1374-1384.

813. Edwards,D.I. (1993) Nitroimidazole drugs--action and resistance mechanisms. I. Mechanisms of action. *J.Antimicrob.Chemother.* **31** (1), 9-20.

814. Jorgensen,M.A., Manos,J., Mendz,G.L., and Hazell,S.L. (1998) The mode of action of metronidazole in Helicobacter pylori: futile cycling or reduction? *J.Antimicrob.Chemother.* **41** (1), 67-75.

815. Goodwin,A., Kersulyte,D., Sisson,G., Veldhuyzen van Zanten,S.J., Berg,D.E., and Hoffman,P.S. (1998) Metronidazole resistance in Helicobacter pylori is due to null mutations in a gene (rdxA) that encodes an oxygen-insensitive NADPH nitroreductase. *Mol.Microbiol* **28** (2), 383-393.

816. Kwon,D.H., Osato,M.S., Graham,D.Y., and el Zaatari,F.A. (2000) Quantitative RT-PCR analysis of multiple genes encoding putative metronidazole nitroreductases from Helicobacter pylori. *Int.J Antimicrob.Agents* **15** (1), 31-36.

817. Hoffman,P.S., Goodwin,A., Johnsen,J., Magee,K., and Veldhuyzen van Zanten,S.J. (1996) Metabolic activities of metronidazole-sensitive and -resistant strains of Helicobacter pylori: repression of pyruvate oxidoreductase and expression of isocitrate lyase activity correlate with resistance. *J.Bacteriol.* **178** (16), 4822-4829.

818. Jeong,J.Y., Mukhopadhyay,A.K., Dailidiene,D., Wang,Y., Velapatino,B., Gilman,R.H., Parkinson,A.J., Nair,G.B., Wong,B.C., Lam,S.K., Mistry,R., Segal,I., Yuan,Y., Gao,H., Alarcon,T., Brea,M.L., Ito,Y., Kersulyte,D., Lee,H.K., Gong,Y., Goodwin,A., Hoffman,P.S., and Berg,D.E. (2000) Sequential inactivation of rdxA (HP0954) and frxA (HP0642) nitroreductase genes causes moderate and high-level metronidazole resistance in Helicobacter pylori. *J Bacteriol.* **182** (18), 5082-5090.

819. Hughes,N.J., Chalk,P.A., Clayton,C.L., and Kelly,D.J. (1995) Identification of carboxylation enzymes and characterization of a novel four-subunit pyruvate:flavodoxin oxidoreductase from Helicobacter pylori. *J Bacteriol.* **177** (14), 3953-3959.

820. Kaihovaara,P., Hook-Nikanne,J., Uusi-Oukari,M., Kosunen,T.U., and Salaspuro,M. (1998) Flavodoxin-dependent pyruvate oxidation, acetate production and metronidazole reduction by Helicobacter pylori. *J Antimicrob.Chemother.* **41** (2), 171-177.

821. Dore,M.P., Graham,D.Y., and Sepulveda,A.R. (1999) Different penicillin-binding protein profiles in amoxicillin-resistant Helicobacter pylori. *Helicobacter.* **4** (3), 154-161.

822. Dore,M.P., Osato,M.S., Realdi,G., Mura,I., Graham,D.Y., and Sepulveda,A.R. (1999) Amoxycillin tolerance in Helicobacter pylori. *J.Antimicrob.Chemother.* **43** (1), 47-54.

823. Kwon,D.H., Dore,M.P., Kim,J.J., Kato,M., Lee,M., Wu,J.Y., and Graham,D.Y. (2003) High-level beta-lactam resistance associated with acquired multidrug resistance in Helicobacter pylori. *Antimicrob.Agents Chemother.* **47** (7), 2169-2178.

824. Horii,T., Mase,K., Suzuki,Y., Kimura,T., Ohta,M., Maekawa,M., Kanno,T., and Kobayashi,M. (2002) Antibacterial activities of beta-lactamase inhibitors associated with morphological changes of cell wall in Helicobacter pylori. *Helicobacter.* **7** (1), 39-45.

825. Moore,R.A., Beckthold,B., Wong,S., Kureishi,A., and Bryan,L.E. (1995) Nucleotide sequence of the gyrA gene and characterization of ciprofloxacin-resistant mutants of Helicobacter pylori. *Antimicrob.Agents Chemother.* **39** (1), 107-111.

826. Heep,M., ., Beck,D., Decker,J., Prohaska,E., Rieger,U., and Lehn,N. (2000) Mutations at four distinct regions of the rpoB gene can reduce the susceptibility of Helicobacter pylori to rifamycins. *Antimicrob.Agents Chemother.* **44** (6), 1713-1715.

827. Trieber,C.A. and Taylor,D.E. (2002) Mutations in the 16S rRNA genes of Helicobacter pylori mediate resistance to tetracycline. *J.Bacteriol.* **184** (8), 2131-2140.

826. Gerrits,M.M., Schuijffel,D., van Zwet,A.A., Kuipers,E.J., Vandenbroucke-Grauls,C.M., an8 Kusters,J.G. (2002) Alterations in penicillin-binding protein 1A confer resistance to beta-lactam antibiotics in Helicobacter pylori. *Antimicrob.Agents Chemother.* **46** (7), 2229-2233.

829. Wermeille,J., Cunningham,M., Dederding,J.P., Girard,L., Baumann,R., Zelger,G., Buri,P., Metry,J.M., Sitavanc,R., Gallaz,L., Merki,H., and Godin,N. (2002) Failure of Helicobacter pylori eradication: is poor compliance the main cause? *Gastroenterol.Clin.Biol.* **26** (3), 216-219.

830. Basset,C., Holton,J., Gatta,L., Ricci,C., Bernabucci,V., Liuzzi,G., and Vaira,D. (2004) Helicobacter pylori infection: anything new should we know? *Aliment.Pharmacol.Ther.* **20 Suppl 2** 31-41.

831. Cowan,M.M. (1999) Plant products as antimicrobial agents. *Clinical Microbiology Reviews* **12** (4), 564-582.

832. O'Mahony,R., AL-Khtheeri,H., Weerasekera,D., Fernando,N., Vaira,D., Holton,J., and Basset,C. (2005) Bactericidal and anti-adhesive properties of culinary and medicinal plants against *Helicobacter pylori. World J Gastroenterol.* **11** (47), 7499-7507.
Ref ID: 2192

833. Clare,D.A., Catignani,G.L., and Swaisgood,H.E. (2003) Biodefense properties of milk: the role of antimicrobial proteins and peptides. *Curr.Pharm.Des* **9** (16), 1239-1255.

834. Gopal,P.K. and Gill,H.S. (2000) Oligosaccharides and glycoconjugates in bovine milk an4 colostrum. *Br.J Nutr.* **84 Suppl 1** S69-S74.

835. van Hooijdonk,A.C., Kussendrager,K.D., and Steijns,J.M. (2000) In vivo antimicrobial an antiviral activity of components in bovine milk and colostrum involved in non-specific defence. *Br.J Nutr.* **84 Suppl 1** S127-S134.

836. Nostro,A., Cellini,L., Di Bartolomeo,S., Di Campli,E., Grande,R., Cannatelli,M.A., Marzio,L., and Alonzo,V. (2005) Antibacterial effect of plant extracts against Helicobacter pylori. *Phytother.Res.* **19** (3), 198-202.

837. al Somal,N., Coley,K.E., Molan,P.C., and Hancock,B.M. (1994) Susceptibility of Helicobacter pylori to the antibacterial activity of manuka honey. *J.R.Soc.Med.* **87** (1), 9-12.

838. Koo,H., Gomes,B.P., Rosalen,P.L., Ambrosano,G.M., Park,Y.K., and Cury,J.A. (2000) In vitro antimicrobial activity of propolis and Arnica montana against oral pathogens. *Arch.Oral Biol.* **45** (2), 141-148.

839. Hiramoto,S., Itoh,K., Shizuuchi,S., Kawachi,Y., Morishita,Y., Nagase,M., Suzuki,Y., Nobuta,Y., Sudou,Y., Nakamura,O., Kagaya,I., Goshima,H., Kodama,Y., Icatro,F.C., Koizumi,W., Saigenji,K., Miura,S., Sugiyama,T., and Kimura,N. (2004) Melanoidin, a food protein-derived advanced maillard reaction product, suppresses Helicobacter pylori in vitro and in vivo. *Helicobacter.* **9** (5), 429-435.

840. Matsumoto,M., Hara,K., Kimata,H., Benno,Y., and Shimamoto,C. (2005) Exfoliation of Helicobacter pylori from gastric mucin by glycopolypeptides from buttermilk. *J.Dairy Sci.* **88** (1), 49-54.

841. Lindequist,U., Niedermeyer,T.H., and Julich,W.D. (2005) The pharmacological potential of mushrooms. *Evid.Based.Complement Alternat.Med.* **2** (3), 285-299.

842. Mercado,L., Schmitt,P., Marshall,S., and Arenas,G. (2005) Gill tissues of the mussel *Mytilus edulis chilensis*: A new source for antimicrobial peptides. *Electronic Journal of Biotechnology [online].* **8** (3).

843. Bernan,V.S., Greenstein,M., and Carter,G.T. (2004) Mining Marine Microorganisms as a Source of New Antimicrobials and Antifungals. *Current Medicinal Chemistry-Anti-infective agents* **3** (3), 181-195.

844. Cooper,E.L., Hrzenjak,T.M., and Grdisa,M. (2004) Alternative sources of fibrinolytic, anticoagulative, antimicrobial and anticancer molecules. *Int.J Immunopathol.Pharmacol.* **17** (3), 237-244.

845. Patrzykat,A., Gallant,J.W., Seo,J.K., Pytyck,J., and Douglas,S.E. (2003) Novel antimicrobial peptides derived from flatfish genes. *Antimicrob.Agents Chemother.* **47** (8), 2464-2470.

846. Marchetti,O., Moreillon,P., Glauser,M.P., Bille,J., and Sanglard,D. (2000) Potent synergism of the combination of fluconazole and cyclosporine in Candida albicans. *Antimicrob.Agents Chemother.* **44** (9), 2373-2381.

847. Lee,M.D., Galazzo,J.L., Staley,A.L., Lee,J.C., Warren,M.S., Fuernkranz,H., Chamberland,S., Lomovskaya,O., and Miller,G.H. (2001) Microbial fermentation-derived inhibitors of efflux-pump-mediated drug resistance. *Farmaco* **56** (1-2), 81-85.

848. Schuetzer-Muehlbauer,M., Willinger,B., Egner,R., Ecker,G., and Kuchler,K. (2003) Reversal of antifungal resistance mediated by ABC efflux pumps from Candida albicans functionally expressed in yeast. *Int.J.Antimicrob.Agents* **22** (3), 291-300.

849. Shukla,S., Sauna,Z.E., Prasad,R., and Ambudkar,S.V. (2004) Disulfiram is a potent modulator of multidrug transporter Cdr1p of Candida albicans. *Biochem.Biophys.Res.Commun.* **322** (2), 520-525.

850. Niimi,K., Harding,D.R., Parshot,R., King,A., Lun,D.J., Decottignies,A., Niimi,M., Lin,S., Cannon,R.D., Goffeau,A., and Monk,B.C. (2004) Chemosensitization of fluconazole resistance in Saccharomyces cerevisiae and pathogenic fungi by a D-octapeptide derivative. *Antimicrob.Agents Chemother.* **48** (4), 1256-1271.

851. Medaglini,D., Rush,C.M., Sestini,P., and Pozzi,G. (1997) Commensal bacteria as vectors for mucosal vaccines against sexually transmitted diseases: vaginal colonization with recombinant streptococci induces local and systemic antibodies in mice. *Vaccine* **15** (12-13), 1330-1337.

852. Midolo,P.D., Lambert,J.R., Hull,R., Luo,F., and Grayson,M.L. (1995) In vitro inhibition of Helicobacter pylori NCTC 11637 by organic acids and lactic acid bacteria. *J.Appl.Bacteriol.* **79** (4), 475-479.

853. Coconnier,M.H., Lievin,V., Hemery,E., and Servin,A.L. (1998) Antagonistic activity against Helicobacter infection in vitro and in vivo by the human Lactobacillus acidophilus strain LB. *Appl.Environ.Microbiol.* **64** (11), 4573-4580.

854. Mastromarino,P., Brigidi,P., Macchia,S., Maggi,L., Pirovano,F., Trinchieri,V., Conte,U., and Matteuzzi,D. (2002) Characterization and selection of vaginal Lactobacillus strains for the preparation of vaginal tablets. *J.Appl.Microbiol.* **93** (5), 884-893.

855. Demidova,T.N. and Hamblin,M.R. (2004) Photodynamic therapy targeted to pathogens. *Int.J Immunopathol.Pharmacol.* **17** (3), 245-254.

856. Conway,B.R. (2005) Drug delivery strategies for the treatment of Helicobacter pylori infections. *Curr.Pharm.Des* **11** (6), 775-790.

857. Higo,S., Ori,K., Takeuchi,H., Yamamoto,H., Hino,T., and Kawashima,Y. (2004) A novel evaluation method of gastric mucoadhesive property in vitro and the mucoadhesive mechanism of tetracycline-sucralfate acidic complex for eradication of Helicobacter pylori. *Pharm.Res.* **21** (3), 413-419.

858. Higo,S., Takeuchi,H., Yamamoto,H., Hino,T., and Kawashima,Y. (2004) The acidic complexation of tetracycline with sucralfate for its mucoadhesive preparation. *Drug Dev.Ind.Pharm.* **30** (7), 715-724.

859. Chang,J.Y., Oh,Y.K., Kong,H.S., Kim,E.J., Jang,D.D., Nam,K.T., and Kim,C.K. (2002) Prolonged antifungal effects of clotrimazole-containing mucoadhesive thermosensitive gels on vaginitis. *J.Control Release* **82** (1), 39-50.

860. Gotoh,A., Akamatsu,T., Shimizu,T., Shimodaira,K., Kaneko,T., Kiyosawa,K., Ishida,K., Ikeno,T., Sugiyama,A., Kawakami,Y., Ota,H., and Katsuyama,T. (2002) Additive effect of pronase on the efficacy of eradication therapy against Helicobacter pylori. *Helicobacter.* **7** (3), 183-191.

861. Vajdy,M., Singh,M., Ugozzoli,M., Briones,M., Soenawan,E., Cuadra,L., Kazzaz,J., Ruggiero,P., Peppoloni,S., Norelli,F., Del Giudice,G., and O'Hagan,D. (2003) Enhanced mucosal and systemic immune responses to Helicobacter pylori antigens through mucosal priming followed by systemic boosting immunizations. *Immunology* **110** (1), 86-94.

862. Del Giudice,G. and Michetti,P. (2004) Inflammation, immunity and vaccines for Helicobacter pylori. *Helicobacter.* **9 Suppl 1** 23-28.

863. Fujii,R., Morihara,F., Fukushima,K., Oku,T., Hifumi,E., and Uda,T. (2004) Recombinant antigen from Helicobacter pylori urease as vaccine against H. pylori-associated disease. *Biotechnol.Bioeng.* **86** (7), 737-746.

864. De Bernardis,F., Santoni,G., Boccanera,M., Spreghini,E., Adriani,D., Morelli,L., and Cassone,A. (2000) Local anticandidal immune responses in a rat model of vaginal infection by and protection against Candida albicans. *Infect.Immun.* **68** (6), 3297-3304.

865. De Bernardis,F., Boccanera,M., Adriani,D., Girolamo,A., and Cassone,A. (2002) Intravaginal and intranasal immunizations are equally effective in inducing vaginal antibodies and conferring protection against vaginal candidiasis. *Infect.Immun.* **70** (5), 2725-2729.

866. Fidel,P.L., Jr., Luo,W., Chabain,J., Wolf,N.A., and Van Buren,E. (1997) Use of cellular depletion analysis to examine circulation of immune effector function between the vagina and the periphery. *Infect.Immun.* **65** (9), 3939-3943.

867. Sobel,J.D. (1992) Pathogenesis and treatment of recurrent vulvovaginal candidiasis. *Clin.Infect.Dis.* **14 Suppl 1** S148-S153.

868. Cassone,A., Boccanera,M., Adriani,D., Santoni,G., and De Bernardis,F. (1995) Rats clearing a vaginal infection by Candida albicans acquire specific, antibody-mediated resistance to vaginal reinfection. *Infect.Immun.* **63** (7), 2619-2624.

869. De Bernardis,F., Boccanera,M., Adriani,D., Spreghini,E., Santoni,G., and Cassone,A. (1997) Protective role of antimannan and anti-aspartyl proteinase antibodies in an experimental model of Candida albicans vaginitis in rats. *Infect.Immun.* **65** (8), 3399-3405.

870. Han,Y., Morrison,R.P., and Cutler,J.E. (1998) A vaccine and monoclonal antibodies that enhance mouse resistance to Candida albicans vaginal infection. *Infect.Immun.* **66** (12), 5771-5776.

871. Sharon,N. and Ofek,I. (2002) Fighting infectious diseases with inhibitors of microbial adhesion to host tissues. *Crit Rev.Food Sci.Nutr.* **42** (3 Suppl), 267-272.

872. Hasegawa,M., Amano,A., Muraoka,H., Kobayashi,I., Kimoto,M., Kato,M., Fujioka,T., and Nasu,M. (2002) [A multicenter study of a new Helicobacter pylori selective medium. Columbia horse blood agar HP]. *Kansenshogaku Zasshi* **76** (5), 341-346.

873. Benaissa,M., Babin,P., Quellard,N., Pezennec,L., Cenatiempo,Y., and Fauchere,J.L. (1996) Changes in Helicobacter pylori ultrastructure and antigens during conversion from the bacillary to the coccoid form. *Infect.Immun.* **64** (6), 2331-2335.

874. Sorberg,M., Nilsson,M., Hanberger,H., and Nilsson,L.E. (1996) Morphologic conversion of Helicobacter pylori from bacillary to coccoid form. *Eur.J.Clin.Microbiol.Infect.Dis.* **15** (3), 216-219.

875. Berry,V., Jennings,K., and Woodnutt,G. (1995) Bactericidal and morphological effects of amoxicillin on Helicobacter pylori. *Antimicrob.Agents Chemother.* **39** (8), 1859-1861.

876. Bode,G., Mauch,F., and Malfertheiner,P. (1993) The coccoid forms of Helicobacter pylori. Criteria for their viability. *Epidemiol.Infect.* **111** (3), 483-490.

877. Catrenich,C.E. and Makin,K.M. (1991) Characterization of the morphologic conversion of Helicobacter pylori from bacillary to coccoid forms. *Scand.J.Gastroenterol.Suppl* **181** 58-64.
878. Young,K.A., Allaker,R.P., and Hardie,J.M. (2001) Morphological analysis of Helicobacter pylori from gastric biopsies and dental plaque by scanning electron microscopy. *Oral Microbiol.Immunol.* **16** (3), 178-181.

879. Chan,W.Y., Hui,P.K., Leung,K.M., Chow,J., Kwok,F., and Ng,C.S. (1994) Coccoid forms of Helicobacter pylori in the human stomach. *Am.J Clin.Pathol.* **102** (4), 503-507.

880. Cole,S.P., Cirillo,D., Kagnoff,M.F., Guiney,D.G., and Eckmann,L. (1997) Coccoid and spiral Helicobacter pylori differ in their abilities to adhere to gastric epithelial cells and induce interleukin-8 secretion. *Infect Immun.* **65** (2), 843-846.

881. Kusters,J.G., Gerrits,M.M., Van Strijp,J.A., and Vandenbroucke-Grauls,C.M. (1997) Coccoid forms of Helicobacter pylori are the morphologic manifestation of cell death. *Infect Immun.* **65** (9), 3672-3679.

882. Nilius,M., Strohle,A., Bode,G., and Malfertheiner,P. (1993) Coccoid like forms (CLF) of Helicobacter pylori. Enzyme activity and antigenicity. *Zentralbl.Bakteriol.* **280** (1-2), 259-272.

883. She,F.F., Lin,J.Y., Liu,J.Y., Huang,C., and Su,D.H. (2003) Virulence of water-induced coccoid Helicobacter pylori and its experimental infection in mice. *World J Gastroenterol.* **9** (3), 516-520.

884. Vijayakumari,S., Khin,M.M., Jiang,B., and Ho,B. (1995) The pathogenic role of the coccoid form of Helicobacter pylori. *Cytobios* **82** (331), 251-260.

885. Osaki,T., Yamaguchi,H., Taguchi,H., Fukada,M., Kawakami,H., Hirano,H., and Kamiya,S. (2002) Interleukin-8 induction and adhesion of the coccoid form of Helicobacter pylori. *J.Med.Microbiol.* **51** (4), 295-299.

886. Jabra-Rizk,M.A., Falkler,W.A., Jr., Merz,W.G., and Meiller,T.F. (2001) New assay for measuring cell surface hydrophobicities of Candida dubliniensis and Candida albicans. *Clin.Diagn.Lab Immunol.* **8** (3), 585-587.

887. Reinhard,J., Basset,C., Holton,J., Binks,M., Youinou,P., and Vaira,D. (2000) Image analysis method to assess adhesion of Helicobacter pylori to gastric epithelium using confocal laser scanning microscopy. *J.Microbiol.Methods* **39** (3), 179-187.

888. BD BioSciences. Introduction to Flow Cytometry: A Learning Guide. 2000. San Jose, California. USA. Ref Type: Pamphlet
889. Fox,C.H., Johnson,F.B., Whiting,J., and Roller,P.P. (1985) Formaldehyde fixation. *J.Histochem.Cytochem.* **33** (8), 845-853.

890. Holgate,C.S., Jackson,P., Pollard,K., Lunny,D., and Bird,C.C. (1986) Effect fixation on T and B lymphocyte surface membrane antigen demonstration in paraffin processed tissue. *J.Pathol.* **149** (4), 293-300.

891. Beckstead,J.H. (1994) A simple technique for preservation of fixation-sensitive antigens in paraffin-embedded tissues. *J.Histochem.Cytochem.* **42** (8), 1127-1134.

892. Gonzalez,L., Anderson,I., Deane,D., Summers,C., and Buxton,D. (2001) Detection of immune system cells in paraffin wax-embedded ovine tissues. *J.Comp Pathol.* **125** (1), 41-47.

893. Shi,S.R., Cote,R.J., and Taylor,C.R. (2001) Antigen retrieval techniques: current perspectives. *J Histochem.Cytochem.* **49** (8), 931-937.

894. Gown,A.M. (2004) Unmasking the mysteries of antigen or epitope retrieval and formalin fixation. *Am.J.Clin.Pathol.* **121** (2), 172-174.

895. Elias,J.M. (2000) Commentary: immunohistochemistry: a brief historical perspective. In: Shi,S.R., Gu,J., and Taylor,C.R. (eds), pp. 7-13, Eaton Publishing, Natick, Massachusetts, USA.

896. Kim,S.H., Kook,M.C., Shin,Y.K., Park,S.H., and Song,H.G. (2004) Evaluation of antigen retrieval buffer systems. *J.Mol.Histol.* **35** (4), 409-416.

897. Sergio,N.F., Cecilia,G.V., Sonia,L., Mora Pedro,G.D., Joaquin,G.E., and Mary,F.M. (2004) Electrochemical antigen-retrieval of formaldehyde fixed and paraffin-embedded archived leprosy skin biopsies. *J.Mol.Histol.* **35** (5), 433-441.

898. Folkvord,J.M., Viders,D., Coleman-Smith,A., and Clark,R.A. (1989) Optimization of immunohistochemical techniques to detect extracellular matrix proteins in fixed skin specimens. *J.Histochem.Cytochem.* **37** (1), 105-113.

899. Kalo,A. and Segal,E. (1988) Interaction of Candida albicans with genital mucosa: effect of sex hormones on adherence of yeasts in vitro. *Can.J.Microbiol.* **34** (3), 224-228.

900. Kinsman,O.S. and Collard,A.E. (1986) Hormonal factors in vaginal candidiasis in rats. *Infect.Immun.* **53** (3), 498-504.

901. Elzay,R.P. (1983) A modification of the Papanicolaou exfoliative cytology stain to demonstrate keratin in paraffin-block tissue sections. *Oral Surg.Oral Med.Oral Pathol.* **56** (1), 51-53.

902. McLean,I.W. and Nakane,P.K. (1974) Periodate-lysine-paraformaldehyde fixative. A new fixation for immunoelectron microscopy. *J.Histochem.Cytochem.* **22** (12), 1077-1083.

903. Bainton,D.F. (1999) Distinct granule populations in human neutrophils and lysosomal organelles identified by immuno-electron microscopy. *J.Immunol.Methods* **232** (1-2), 153-168.

904. Chayen,J. and Bitensky,L. (1991) *Practical Histochemistry.*, John Wiley and Sons, West Sussex, UK.

905. O'Mahony,R., Basset,C., Holton,J., Vaira,D., and Roitt,I. (2005) Comparison of image analysis software packages in the assessment of adhesion of microorganisms to mucosal epithelium using confocal laser scanning microscopy. *J.Microbiol.Methods* **61** (1), 105-126.

906. Tobin,K.J., Onstott,T.C., DeFlaun,M.F., Colwell,F.S., and Fredrickson,J. (1999) In situ imaging of microorganisms in geologic material. *J.Microbiol.Methods* **37** (3), 201-213.

907. Hood,S.K. and Zottola,E.A. (1997) Adherence to stainless steel by foodborne microorganisms during growth in model food systems. *Int.J.Food Microbiol.* **37** (2-3), 145-153.

908. Williamson,F.A. and Palframan,K.R. (1989) An improved method for collecting and staining microorganisms for enumeration by fluorescence light microscopy. *J.Microsc.* **154 (Pt 3)** 267-272.

909. Pernthaler,J., Pernthaler,A., and Amann,R. (2003) Automated enumeration of groups of marine picoplankton after fluorescence in situ hybridization. *Appl.Environ.Microbiol.* **69** (5), 2631-2637.

910. Pons,M.N. and Vivier,H. (2000) Biomass quantification by image analysis. *Adv.Biochem.Eng Biotechnol.* **66** 133-184.

911. Gualtieri,P., Ghetti,F., Passarelli,V., and Barsanti,L. (1988) Microorganism track reconstruction: an image processing approach. *Comput.Biol.Med.* **18** (2), 57-63.

912. Goldstein,E., Donovan,R.M., and Kim,Y. (1988) Applications of computerized microscopic image analysis in infectious diseases. *Rev.Infect.Dis.* **10** (1), 92-102.

913. Corkidi,G., Diaz-Uribe,R., Folch-Mallol,J.L., and Nieto-Sotelo,J. (1998) COVASIAM: an image analysis method that allows detection of confluent microbial colonies and colonies of various sizes for automated counting. *Appl.Environ.Microbiol.* **64** (4), 1400-1404.

914. Sjollema,J., van der Mei,H.C., Uyen,H.M., and Busscher,H.J. (1990) Direct observations of cooperative effects in oral streptococcal adhesion to glass by analysis of the spatial arrangement of adhering bacteria. *FEMS Microbiol.Lett.* **57** (3), 263-269.

915. Barthelson,R., Hopkins,C., and Mobasseri,A. (1999) Quantitation of bacterial adherence by image analysis. *J.Microbiol.Methods* **38** (1-2), 17-23.

916. Filoche,S.K., Zhu,M., and Wu,C.D. (2004) In situ biofilm formation by multi-species oral bacteria under flowing and anaerobic conditions. *J.Dent.Res.* **83** (10), 802-806.

917. Monier,J.M. and Lindow,S.E. (2004) Frequency, size, and localization of bacterial aggregates on bean leaf surfaces. *Appl.Environ.Microbiol.* **70** (1), 346-355.

918. Midelet,G. and Carpentier,B. (2004) Impact of cleaning and disinfection agents on biofilm structure and on microbial transfer to a solid model food. *J.Appl.Microbiol.* **97** (2), 262-270.

919. Niu,C. and Gilbert,E.S. (2004) Colorimetric method for identifying plant essential oil components that affect biofilm formation and structure. *Appl.Environ.Microbiol.* **70** (12), 6951-6956.

920. Wilson,S., Hamilton,M.A., Hamilton,G.C., Schumann,M.R., and Stoodley,P. (2004) Statistical quantification of detachment rates and size distributions of cell clumps from wild-type (PAO1) and cell signaling mutant (JP1) Pseudomonas aeruginosa biofilms. *Appl.Environ.Microbiol.* **70** (10), 5847-5852.

921. Schonholzer,F., Hahn,D., Zarda,B., and Zeyer,J. (2002) Automated image analysis and in situ hybridization as tools to study bacterial populations in food resources, gut and cast of Lumbricus terrestris L. *J.Microbiol.Methods* **48** (1), 53-68.

922. Jansen,G.J., Wildeboer-Veloo,A.C., Tonk,R.H., Franks,A.H., and Welling,G.W. (1999) Development and validation of an automated, microscopy-based method for enumeration of groups of intestinal bacteria. *J.Microbiol.Methods* **37** (3), 215-221.

923. Wit,P. and Busscher,H. (1998) Application of an artificial neural network in the enumeration of yeasts and bacteria adhering to solid substrata. *Journal of Microbiological Methods* **32** 281-290.

924. Jespers,L., Schon,O., Famm,K., and Winter,G. (2004) Aggregation-resistant domain antibodies selected on phage by heat denaturation. *Nat.Biotechnol.* **22** (9), 1161-1165.

925. Jespers,L., Schon,O., James,L.C., Veprintsev,D., and Winter,G. (2004) Crystal structure of HEL4, a soluble, refoldable human V(H) single domain with a germ-line scaffold. *J.Mol.Biol.* **337** (4), 893-903.

926. Holliger,P. and Hudson,P.J. (2005) Engineered antibody fragments and the rise of single domains. *Nat.Biotechnol.* **23** (9), 1126-1136.

927. Oggioni,M.R., Beninati,C., Boccanera,M., Medaglini,D., Spinosa,M.R., Maggi,T., Conti,S., Magliani,W., De Bernardis,F., Teti,G., Cassone,A., Pozzi,G., and Polonelli,L. (2001) Recombinant Streptococcus gordonii for mucosal delivery of a scFv microbicidal antibody. *Int.Rev.Immunol.* **20** (2), 275-287.

928. Polonelli,L., Magliani,W., Conti,S., Bracci,L., Lozzi,L., Neri,P., Adriani,D., De Bernardis,F., and Cassone,A. (2003) Therapeutic activity of an engineered synthetic killer antiidiotypic antibody fragment against experimental mucosal and systemic candidiasis. *Infect Immun.* **71** (11), 6205-6212.

929. McCafferty,J., Griffiths,A.D., Winter,G., and Chiswell,D.J. (1990) Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* **348** (6301), 552-554.

930. Abu-el Teen,K., Ghannum,M., and Stretton,R.J. (1989) Effects of sub-inhibitory concentrations of antifungal agents on adherence of Candida spp. to buccal epithelial cells in vitro. *Mycoses* **32** (11), 551-562.

931. Braga,P.C., Dal Sasso,M., Maci,S., Piatti,G., Dannhorn,D.R., and Bohn,M. (1995) Inhibition of Candida albicans adhesiveness to human buccal and vaginal cells by sub-inhibitory concentrations of rilopirox. *Arzneimittelforschung.* **45** (1), 84-87.

932. Jones,D.S., McGovern,J.G., Woolfson,A.D., and Gorman,S.P. (1997) The effects of hexetidine (Oraldene) on the adherence of Candida albicans to human buccal epithelial cells in vitro and ex vivo and on in vitro morphogenesis. *Pharm.Res.* **14** (12), 1765-1771.

933. Ellepola,A.N. and Samaranayake,L.P. (1998) The effect of limited exposure to antimycotics on the relative cell-surface hydrophobicity and the adhesion of oral Candida albicans to buccal epithelial cells. *Arch.Oral Biol.* **43** (11), 879-887.

934. Fekete-Forgacs,K., Kis,B., Nagy,G., and Lenkey,B. (1999) Effect of fluconazole on the growth and adhesion of Candida albicans in the presence of antineoplastic agents. *J.Basic Microbiol.* **39** (5-6), 305-310.

935. Borg-von Zepelin,M., Zaschke,K., Gross,U., Monod,M., and Muller,F.M. (2002) Effect of Micafungin (FK463) on Candida albicans adherence to epithelial cells. *Chemotherapy* **48** (3), 148-153.

936. Dorocka-Bobkowska,B., Konopka,K., and Duzgunes,N. (2003) Influence of antifungal polyenes on the adhesion of Candida albicans and Candida glabrata to human epithelial cells in vitro. *Arch.Oral Biol.* **48** (12), 805-814.

937. Bektic,J., Lell,C.P., Fuchs,A., Stoiber,H., Speth,C., Lass-Florl,C., Borg-von Zepelin,M., Dierich,M.P., and Wurzner,R. (2001) HIV protease inhibitors attenuate adherence of Candida albicans to epithelial cells in vitro. *FEMS Immunol.Med.Microbiol.* **31** (1), 65-71.

938. Borg-von Zepelin,M., Meyer,I., Thomssen,R., Wurzner,R., Sanglard,D., Telenti,A., and Monod,M. (1999) HIV-Protease inhibitors reduce cell adherence of Candida albicans strains by inhibition of yeast secreted aspartic proteases. *J.Invest Dermatol.* **113** (5), 747-751.

939. McCarron,P.A., Donnelly,R.F., Canning,P.E., McGovern,J.G., and Jones,D.S. (2004) Bioadhesive, non-drug-loaded nanoparticles as modulators of candidal adherence to buccal epithelial cells: a potentially novel prophylaxis for candidosis. *Biomaterials* **25** (12), 2399-2407.

940. Moragues,M.D., Omaetxebarria,M.J., Elguezabal,N., Sevilla,M.J., Conti,S., Polonelli,L., and Ponton,J. (2003) A monoclonal antibody directed against a Candida albicans cell wall mannoprotein exerts three anti-C. albicans activities. *Infect.Immun.* **71** (9), 5273-5279.

941. Umazume,M., Ueta,E., and Osaki,T. (1995) Reduced inhibition of Candida albicans adhesion by saliva from patients receiving oral cancer therapy. *J.Clin.Microbiol.* **33** (2), 432-439.

942. D'Auria,F.D., Tecca,M., Scazzocchio,F., Renzini,V., and Strippoli,V. (2003) Effect of propolis on virulence factors of Candida albicans. *J.Chemother.* **15** (5), 454-460.

943. Abu-Elteen,K.H. (2000) Effects of date extract on adhesion of Candida species to human buccal epithelial cells in vitro. *J.Oral Pathol.Med.* **29** (5), 200-205.

944. Taweechaisupapong,S., Choopan,T., Singhara,S., Chatrchaiwiwatana,S., and Wongkham,S. (2005) In vitro inhibitory effect of Streblus asper leaf-extract on adhesion of Candida albicans to human buccal epithelial cells. *J.Ethnopharmacol.* **96** (1-2), 221-226.

945. Goran,D., Vesna,A., Adela,S., Biljana,T.K., and Snezana,M. (2004) Polygynax in the treatment of fungal and non specific vaginitis. *Akush.Ginekol.(Sofiia)* **43** (6), 23-26.

946. Petraitis,V., Petraitiene,R., Groll,A.H., Roussillon,K., Hemmings,M., Lyman,C.A., Sein,T., Bacher,J., Bekersky,I., and Walsh,T.J. (2002) Comparative antifungal activities and plasma pharmacokinetics of micafungin (FK463) against disseminated candidiasis and invasive pulmonary aspergillosis in persistently neutropenic rabbits. *Antimicrob.Agents Chemother.* **46** (6), 1857-1869.

947. Foldvari,M., Jaafari,M.R., Radhi,J., and Segal,D. (2005) Efficacy of the antiadhesin octyl O-(2-acetamido-2-deoxy-beta-D-galactopyranosyl)-(1-4)-2-O-propyl-beta-D-ga lactopyranoside (Fimbrigal-P) in a rat oral candidiasis model. *Antimicrob.Agents Chemother.* **49** (7), 2887-2894.

948. Santangelo,R., Paderu,P., Delmas,G., Chen,Z.W., Mannino,R., Zarif,L., and Perlin,D.S. (2000) Efficacy of oral cochleate-amphotericin B in a mouse model of systemic candidiasis. *Antimicrob.Agents Chemother.* **44** (9), 2356-2360.

949. Domantis Ltd. Domantis: The Domain Antibody Company. 2004. Domantis Ltd, Cambridge, UK.
Ref Type: Pamphlet

950. De Bernardis, F. Human Hypervariable Antibody Domains Recognizing Virulence Traits of *Candida albicans* Confer Passive Protection Against Experimental Vaginal Candidiasis . Lui, H., O'Mahony, R., Basset, C, Holton, J., Roitt, I., and Cassone, A. Nature Biotechnology (Submitted 2005) .

951. Centres for Disease Control Website.
(http://www.cdc/gov/mmwr/preview/mmwrhtml/rr5106a1.htm).

952. MacFarlane,T. (1990) Ecology and epidemiology of *Candida*. In: Samaranayake,L.P. and MacFarlane,T.W. (eds), pp. 21-46, Wright, London, UK.

953. San Millan,R., Elguezabal,N., Regulez,P., Moragues,M.D., Quindos,G., and Ponton,J. (2000) Effect of salivary secretory IgA on the adhesion of Candida albicans to polystyrene. *Microbiology* **146 (Pt 9)** 2105-2112.

954. San Millan,R., Ezkurra,P.A., Quindos,G., Robert,R., Senet,J.M., and Ponton,J. (1996) Effect of monoclonal antibodies directed against Candida albicans cell wall antigens on the adhesion of the fungus to polystyrene. *Microbiology* **142 (Pt 8)** 2271-2277.

955. Becker,K.F. and Hofler,H. (2001) Mutant cell surface receptors as targets for individualized cancer diagnosis and therapy. *Curr.Cancer Drug Targets.* **1** (2), 121-128.

956. Opekun,A.R., El Zaimaity,H.M., Osato,M.S., Gilger,M.A., Malaty,H.M., Terry,M., Headon,D.R., and Graham,D.Y. (1999) Novel therapies for Helicobacter pylori infection. *Aliment.Pharmacol.Ther.* **13** (1), 35-42.

957. Michetti,P., Dorta,G., Wiesel,P.H., Brassart,D., Verdu,E., Herranz,M., Felley,C., Porta,N., Rouvet,M., Blum,A.L., and Corthesy-Theulaz,I. (1999) Effect of whey-based culture supernatant of Lactobacillus acidophilus (johnsonii) La1 on Helicobacter pylori infection in humans. *Digestion* **60** (3), 203-209.

958. Sakamoto,I., Igarashi,M., Kimura,K., Takagi,A., Miwa,T., and Koga,Y. (2001) Suppressive effect of Lactobacillus gasseri OLL 2716 (LG21) on Helicobacter pylori infection in humans. *J.Antimicrob.Chemother.* **47** (5), 709-710.

959. Casswall,T.H., Nilsson,H.O., Bjorck,L., Sjostedt,S., Xu,L., Nord,C.K., Boren,T., Wadstrom,T., and Hammarstrom,L. (2002) Bovine anti-Helicobacter pylori antibodies for oral immunotherapy. *Scand.J.Gastroenterol.* **37** (12), 1380-1385.

960. Casswall,T.H., Sarker,S.A., Albert,M.J., Fuchs,G.J., Bergstrom,M., Bjorck,L., and Hammarstrom,L. (1998) Treatment of Helicobacter pylori infection in infants in rural Bangladesh with oral immunoglobulins from hyperimmune bovine colostrum. *Aliment.Pharmacol.Ther.* **12** (6), 563-568.

961. Parente,F., Cucino,C., Anderloni,A., Grandinetti,G., and Porro,G.B. (2003) Treatment of Helicobacter pylori infection using a novel antiadhesion compound (3'sialyllactose sodium salt). A double blind, placebo-controlled clinical study. *Helicobacter.* **8** (4), 252-256.

962. Gustafsson,A., Hultberg,A., Sjostrom,R., Kacskovics,I., Breimer,M.E., Boren,T., Hammarstrom,L., and Holgersson,J. (2006) Carbohydrate-dependent inhibition of Helicobacter pylori colonization using porcine milk. *Glycobiology* **16** (1), 1-10.

963. Evans,D.G., Evans,D.J., Jr., and Graham,D.Y. (1989) Receptor-mediated adherence of Campylobacter pylori to mouse Y-1 adrenal cell monolayers. *Infect Immun.* **57** (8), 2272-2278.

964. Hata,Y., Murakami,M., and Okabe,S. (2004) Glycoconjugates with NeuAc-NeuAc-Gal-Glc are more effective at preventing adhesion of Helicobacter pylori to gastric epithelial cells than glycoconjugates with NeuAc-Gal-Glc. *J.Physiol Pharmacol.* **55** (3), 607-625.

965. Umamaheshwari,R.B. and Jain,N.K. (2004) Receptor-mediated targeting of lipobeads bearing acetohydroxamic acid for eradication of Helicobacter pylori. *J.Control Release* **99** (1), 27-40.

966. Huesca,M., Gold,B., Sherman,P., Lewin,P., and Lingwood,C. (1993) Therapeutics used to alleviate peptic ulcers inhibit H. pylori receptor binding in vitro. *Zentralbl.Bakteriol.* **280** (1-2), 244-252.

967. Mukai,T., Asasaka,T., Sato,E., Mori,K., Matsumoto,M., and Ohori,H. (2002) Inhibition of binding of Helicobacter pylori to the glycolipid receptors by probiotic Lactobacillus reuteri. *FEMS Immunol.Med.Microbiol.* **32** (2), 105-110.

968. Osaki,T., Yamaguchi,H., Taguchi,H., Fukuda,M., Kawakami,H., Hirano,H., Watanabe,S., Takagi,A., and Kamiya,S. (1998) Establishment and characterisation of a monoclonal antibody to inhibit adhesion of Helicobacter pylori to gastric epithelial cells. *J.Med.Microbiol.* **47** (6), 505-512.

969. Shimamoto,C., Tokioka,S., Hirata,I., Tani,H., Ohishi,H., and Katsu,K. (2002) Inhibition of Helicobacter pylori infection by orally administered yolk-derived anti-Helicobacter pylori antibody. *Hepatogastroenterology* **49** (45), 709-714.

970. Wang,X., Hirmo,S., Willen,R., and Wadstrom,T. (2001) Inhibition of Helicobacter pylori infection by bovine milk glycoconjugates in a BAlb/cA mouse model. *J.Med.Microbiol.* **50** (5), 430-435.

971. Tsai,C.C., Huang,L.F., Lin,C.C., and Tsen,H.Y. (2004) Antagonistic activity against Helicobacter pylori infection in vitro by a strain of Enterococcus faecium TM39. *Int.J.Food Microbiol.* **96** (1), 1-12.

972. Cao,J., Sun,Y., Berglindh,T., Mellgard,B., Li,Z., Mardh,B., and Mardh,S. (2000) Helicobacter pylori-antigen-binding fragments expressed on the filamentous M13 phage prevent bacterial growth. *Biochim.Biophys.Acta* **1474** (1), 107-113.

973. Hayashi,S., Sugiyama,T., Yachi,A., Yokota,K., Hirai,Y., Oguma,K., and Fujii,N. (1997) Effect of ecabet sodium on Helicobacter pylori adhesion to gastric epithelial cells. *J.Gastroenterol.* **32** (5), 593-597.

974. Hayashi,S., Sugiyama,T., Yokota,K., Isogai,H., Isogai,E., Shimomura,H., Oguma,K., Asaka,M., and Hirai,Y. (2000) Combined effect of rebamipide and ecabet sodium on Helicobacter pylori adhesion to gastric epithelial cells. *Microbiol.Immunol.* **44** (7), 557-562.

975. Hayashi,S., Sugiyama,T., Amano,K., Isogai,H., Isogai,E., Aihara,M., Kikuchi,M., Asaka,M., Yokota,K., Oguma,K., Fujii,N., and Hirai,Y. (1998) Effect of rebamipide, a novel antiulcer agent, on Helicobacter pylori adhesion to gastric epithelial cells. *Antimicrob.Agents Chemother.* **42** (8), 1895-1899.

976. Clyne,M., Thomas,J., Weaver,L., and Drumm,B. (1997) In vitro evaluation of the role of antibodies against Helicobacter pylori in inhibiting adherence of the organism to gastric cells. *Gut* **40** (6), 731-738.

977. Wada,T., Aiba,Y., Shimizu,K., Takagi,A., Miwa,T., and Koga,Y. (1999) The therapeutic effect of bovine lactoferrin in the host infected with Helicobacter pylori. *Scand.J.Gastroenterol.* **34** (3), 238-243.

978. Wagner,S., Beil,W., Mai,U.E., Bokemeyer,C., Meyer,H.J., and Manns,M.P. (1994) Interaction between Helicobacter pylori and human gastric epithelial cells in culture: effect of antiulcer drugs. *Pharmacology* **49** (4), 226-237.

979. Alkout,A.M., Blackwell,C.C., Weir,D.M., Poxton,I.R., Elton,R.A., Luman,W., and Palmer,K. (1997) Isolation of a cell surface component of Helicobacter pylori that binds H type 2, Lewis(a), and Lewis(b) antigens. *Gastroenterology* **112** (4), 1179-1187.

980. Stromqvist,M., Falk,P., Bergstrom,S., Hansson,L., Lonnerdal,B., Normark,S., and Hernell,O. (1995) Human milk kappa-casein and inhibition of Helicobacter pylori adhesion to human gastric mucosa. *J.Pediatr.Gastroenterol.Nutr.* **21** (3), 288-296.

981. Ota,H., Nakayama,J., Momose,M., Hayama,M., Akamatsu,T., Katsuyama,T., Graham,D.Y., and Genta,R.M. (1998) Helicobacter pylori infection produces reversible glycosylation changes to gastric mucins. *Virchows Arch.* **433** (5), 419-426.

982. Linden,S., Boren,T., Dubois,A., and Carlstedt,I. (2004) Rhesus monkey gastric mucins: oligomeric structure, glycoforms and Helicobacter pylori binding. *Biochem.J.* **379** (Pt 3), 765-775.

983. Madrid,J.F., Ballesta,J., Castells,M.T., and Hernandez,F. (1990) Glycoconjugate distribution in the human fundic mucosa revealed by lectin- and glycoprotein-gold cytochemistry. *Histochemistry* **95** (2), 179-187.

984. Evans,D.J., Jr., Evans,D.G., Smith,K.E., and Graham,D.Y. (1989) Serum antibody responses to the N-acetylneuraminyllactose-binding hemagglutinin of Campylobacter pylori. *Infect.Immun.* **57** (3), 664-667.

985. Nakamura,T., Kawase,H., Kimura,K., Watanabe,Y., Ohtani,M., Arai,I., and Urashima,T. (2003) Concentrations of sialyloligosaccharides in bovine colostrum and milk during the prepartum and early lactation. *J Dairy Sci.* **86** (4), 1315-1320.

986. Bai,Y., Zhang,Y.L., Wang,J.D., Lin,H.J., Zhang,Z.S., and Zhou,D.Y. (2002) Conservative region of the genes encoding four adhesins of Helicobacter pylori: cloning, sequence analysis and biological information analysis. *Di Yi.Jun.Yi.Da.Xue.Xue.Bao.* **22** (10), 869-871.

987. Lampe,J.W. (1999) Health effects of vegetables and fruit: assessing mechanisms of action in human experimental studies. *Am.J.Clin.Nutr.* **70** (3 Suppl), 475S-490S.

988. Jonkers,D., van den,B.E., van,D., I, Thijs,C., Dorant,E., Hageman,G., and Stobberingh,E. (1999) Antibacterial effect of garlic and omeprazole on Helicobacter pylori. *J.Antimicrob.Chemother.* **43** (6), 837-839.

989. Iimuro,M., Shibata,H., Kawamori,T., Matsumoto,T., Arakawa,T., Sugimura,T., and Wakabayashi,K. (2002) Suppressive effects of garlic extract on Helicobacter pylori-induced gastritis in Mongolian gerbils. *Cancer Lett.* **187** (1-2), 61-68.

990. Sivam,G.P., Lampe,J.W., Ulness,B., Swanzy,S.R., and Potter,J.D. (1997) Helicobacter pylori--in vitro susceptibility to garlic (Allium sativum) extract. *Nutr.Cancer* **27** (2), 118-121.

991. Matsubara,S., Shibata,H., Ishikawa,F., Yokokura,T., Takahashi,M., Sugimura,T., and Wakabayashi,K. (2003) Suppression of Helicobacter pylori-induced gastritis by green tea extract in Mongolian gerbils. *Biochem.Biophys.Res.Commun.* **310** (3), 715-719.

992. Tabak,M., Armon,R., Potasman,I., and Neeman,I. (1996) In vitro inhibition of Helicobacter pylori by extracts of thyme. *J.Appl.Bacteriol.* **80** (6), 667-672.

993. Chatterjee,A., Yasmin,T., Bagchi,D., and Stohs,S.J. (2004) Inhibition of Helicobacter pylori in vitro by various berry extracts, with enhanced susceptibility to clarithromycin. *Mol.Cell Biochem.* **265** (1-2), 19-26.

994. Nariman,F., Eftekhar,F., Habibi,Z., and Falsafi,T. (2004) Anti-Helicobacter pylori activities of six Iranian plants. *Helicobacter.* **9** (2), 146-151.

995. Imai,H., Osawa,K., Yasuda,H., Hamashima,H., Arai,T., and Sasatsu,M. (2001) Inhibition by the essential oils of peppermint and spearmint of the growth of pathogenic bacteria. *Microbios* **106 Suppl 1** 31-39.

996. Martin,K.W. and Ernst,E. (2003) Herbal medicines for treatment of bacterial infections: a review of controlled clinical trials. *J.Antimicrob.Chemother.* **51** (2), 241-246.

997. Galan,M.V., Kishan,A.A., and Silverman,A.L. (2004) Oral broccoli sprouts for the treatment of Helicobacter pylori infection: a preliminary report. *Dig.Dis.Sci.* **49** (7-8), 1088-1090.

998. Burger,O., Ofek,I., Tabak,M., Weiss,E.I., Sharon,N., and Neeman,I. (2000) A high molecular mass constituent of cranberry juice inhibits helicobacter pylori adhesion to human gastric mucus. *FEMS Immunol.Med.Microbiol.* **29** (4), 295-301.

999. Shibata,H., KimuraTakagi,I., Nagaoka,M., Hashimoto,S., Sawada,H., Ueyama,S., and Yokokura,T. (1999) Inhibitory effect of Cladosiphon fucoidan on the adhesion of Helicobacter pylori to human gastric cells. *J.Nutr.Sci.Vitaminol.(Tokyo)* **45** (3), 325-336.

1000. Shibata,H., Iimuro,M., Uchiya,N., Kawamori,T., Nagaoka,M., Ueyama,S., Hashimoto,S., Yokokura,T., Sugimura,T., and Wakabayashi,K. (2003) Preventive effects of Cladosiphon fucoidan against Helicobacter pylori infection in Mongolian gerbils. *Helicobacter.* **8** (1), 59-65.

1001 Lengsfeld,C., Titgemeyer,F., Faller,G., and Hensel,A. (2004) Glycosylated compounds from okra inhibit adhesion of Helicobacter pylori to human gastric mucosa. *J.Agric.Food Chem.* **52** (6), 1495-1503.

1002. Lengsfeld,C., Deters,A., Faller,G., and Hensel,A. (2004) High molecular weight polysaccharides from black currant seeds inhibit adhesion of Helicobacter pylori to human gastric mucosa. *Planta Med.* **70** (7), 620-626.

1003. Lee,J.H., Park,E.K., Uhm,C.S., Chung,M.S., and Kim,K.H. (2004) Inhibition of Helicobacter pylori adhesion to human gastric adenocarcinoma epithelial cells by acidic polysaccharides from Artemisia capillaris and Panax ginseng. *Planta Med.* **70** (7), 615-619.

1004. Megraud,F., Trimoulet,p., Lamouliatte,H., and Boyanova,L. (1991) Bactericidal effect of amoxicillin on Helicobacter pylori in an in vitro model using epithelial cells. *Antimicrob.Agents Chemother.* **35** (5), 869-872.

1005. Sorberg,M., Hanberger,H., Nilsson,M., and Nilsson,L.E. (1997) Pharmacodynamic effects of antibiotics and acid pump inhibitors on Helicobacter pylori. *Antimicrob.Agents Chemother.* **41** (10), 2218-2223.

1006. Sjostrom,J.E., Kuhler,T., and Larsson,H. (1997) Basis for the selective antibacterial activity in vitro of proton pump inhibitors against Helicobacter spp. *Antimicrob.Agents Chemother.* **41** (8), 1797-1801.

1007. Hassan,I.J., Stark,R.M., Greenman,J., and Millar,M.R. (1999) Activities of beta-lactams and macrolides against Helicobacter pylori. *Antimicrob.Agents Chemother.* **43** (6), 1387-1392.

1008. Verdrengh,M., Collins,L.V., Bergin,P., and Tarkowski,A. (2004) Phytoestrogen genistein as an anti-staphylococcal agent. *Microbes.Infect* **6** (1), 86-92.

1009. Canizares,P., Gracia,I., Gomez,L.A., Martin, .A., Boixeda,D., Garcia,A., and de Rafael,L. (2004) Allyl-thiosulfinates, the bacteriostatic compounds of garlic against Helicobacter pylori. *Biotechnol.Prog.* **20** (1), 397-401.

1010. Canizares,P., Gracia,I., Gomez,L.A., Garcia,A., Martin,d.A., Boixeda,D., and de Rafael,L. (2004) Thermal degradation of allicin in garlic extracts and its implication on the inhibition of the in-vitro growth of Helicobacter pylori. *Biotechnol.Prog.* **20** (1), 32-37.

1011. Randhir,R., Lin,Y.T., and Shetty,K. (2004) Phenolics, their antioxidant and antimicrobial activity in dark germinated fenugreek sprouts in response to peptide and phytochemical elicitors. *Asia Pac.J.Clin.Nutr.* **13** (3), 295-307.

1012. Takabayashi,F., Harada,N., Yamada,M., Murohisa,B., and Oguni,I. (2004) Inhibitory effect of green tea catechins in combination with sucralfate on Helicobacter pylori infection in Mongolian gerbils. *J.Gastroenterol.* **39** (1), 61-63.

1013. Yee,Y.K., Koo,M.W., and Szeto,M.L. (2002) Chinese tea consumption and lower risk of Helicobacter infection. *J.Gastroenterol.Hepatol.* **17** (5), 552-555.

1014. Bhamarapravati,S., Pendland,S.L., and Mahady,G.B. (2003) Extracts of spice and food plants from Thai traditional medicine inhibit the growth of the human carcinogen Helicobacter pylori. *In Vivo* **17** (6), 541-544.

1015. Wang,Y.C. and Huang,T.L. (2005) Screening of anti-Helicobacter pylori herbs deriving from Taiwanese folk medicinal plants. *FEMS Immunol.Med.Microbiol.* **43** (2), 295-300.

1016. Tabak,M., Armon,R., and Neeman,I. (1999) Cinnamon extracts' inhibitory effect on Helicobacter pylori. *J.Ethnopharmacol.* **67** (3), 269-277.

1017. Nir,Y., Potasman,I., Stermer,E., Tabak,M., and Neeman,I. (2000) Controlled trial of the effect of cinnamon extract on Helicobacter pylori. *Helicobacter.* **5** (2), 94-97.

1018. Mahady,G.B., Pendland,S.L., Yun,G., and Lu,Z.Z. (2002) Turmeric (Curcuma longa) and curcumin inhibit the growth of Helicobacter pylori, a group 1 carcinogen. *Anticancer Res.* **22** (6C), 4179-4181.

1019. Jones,N.L., Shabib,S., and Sherman,P.M. (1997) Capsaicin as an inhibitor of the growth of the gastric pathogen Helicobacter pylori. *FEMS Microbiol.Lett.* **146** (2), 223-227.

1020. Mahady,G.B., Pendland,S.L., Yun,G.S., Lu,Z.Z., and Stoia,A. (2003) Ginger (Zingiber officinale Roscoe) and the gingerols inhibit the growth of Cag A+ strains of Helicobacter pylori. *Anticancer Res.* **23** (5A), 3699-3702.

1021. Fukai,T., Marumo,A., Kaitou,K., Kanda,T., Terada,S., and Nomura,T. (2002) Anti-Helicobacter pylori flavonoids from licorice extract. *Life Sci.* **71** (12), 1449-1463.

1022. Krausse,R., Bielenberg,J., Blaschek,W., and Ullmann,U. (2004) In vitro anti-Helicobacter pylori activity of Extractum liquiritiae, glycyrrhizin and its metabolites. *J.Antimicrob.Chemother.* **54** (1), 243-246.

1023. Azuine,M.A. and Bhide,S.V. (1992) Chemopreventive effect of turmeric against stomach and skin tumors induced by chemical carcinogens in Swiss mice. *Nutr.Cancer* **17** (1), 77-83.

1024. Huang,M.T., Lou,Y.R., Ma,W., Newmark,H.L., Reuhl,K.R., and Conney,A.H. (1994) Inhibitory effects of dietary curcumin on forestomach, duodenal, and colon carcinogenesis in mice. *Cancer Res.* **54** (22), 5841-5847.

1025. Yamahara,J., Mochizuki,M., Rong,H.Q., Matsuda,H., and Fujimura,H. (1988) The anti-ulcer effect in rats of ginger constituents. *J.Ethnopharmacol.* **23** (2-3), 299-304.

1026. Yoshikawa,M., Yamaguchi,S., Kunimi,K., Matsuda,H., Okuno,Y., Yamahara,J., and Murakami,N. (1994) Stomachic principles in ginger. III. An anti-ulcer principle, 6-gingesulfonic acid, and three monoacyldigalactosylglycerols, gingerglycolipids A, B, and C, from Zingiberis Rhizoma originating in Taiwan. *Chem.Pharm.Bull.(Tokyo)* **42** (6), 1226-1230.

1027. Aruna,K. and Sivaramakrishnan,V.M. (1992) Anticarcinogenic effects of some Indian plant products. *Food Chem.Toxicol.* **30** (11), 953-956.

1028. Gagandeep, Dhanalakshmi,S., Mendiz,E., Rao,A.R., and Kale,R.K. (2003) Chemopreventive effects of Cuminum cyminum in chemically induced forestomach and uterine cervix tumors in murine model systems. *Nutr.Cancer* **47** (2), 171-180.

1029. Gonzalez,C.A., Sanz,J.M., Marcos,G., Pita,S., Brullet,E., Saigi,E., Badia,A., Agudo,A., and Riboli,E. (1993) Borage consumption as a possible gastric cancer protective factor. *Cancer Epidemiol.Biomarkers Prev.* **2** (2), 157-158.

1030. Dehpour,A.R., Zolfaghari,M.E., Samadian,T., and Vahedi,Y. (1994) The protective effect of liquorice components and their derivatives against gastric ulcer induced by aspirin in rats. *J.Pharm.Pharmacol.* **46** (2), 148-149.

1031. Al Howiriny,T., Al Sohaibani,M., El Tahir,K., and Rafatullah,S. (2003) Prevention of experimentally-induced gastric ulcers in rats by an ethanolic extract of "Parsley" Petroselinum crispum. *Am.J.Chin Med.* **31** (5), 699-711.

1032. Lopez-Carrillo,L., Lopez-Cervantes,M., Robles-Diaz,G., Ramirez-Espitia,A., Mohar-Betancourt,A., Meneses-Garcia,A., Lopez-Vidal,Y., and Blair,A. (2003) Capsaicin consumption, Helicobacter pylori positivity and gastric cancer in Mexico. *Int.J.Cancer* **106** (2), 277-282.

1033. Shmuely,H., Burger,O., Neeman,I., Yahav,J., Samra,Z., Niv,Y., Sharon,N., Weiss,E., Athamna,A., Tabak,M., and Ofek,I. (2004) Susceptibility of Helicobacter pylori isolates to the antiadhesion activity of a high-molecular-weight constituent of cranberry. *Diagn.Microbiol.Infect.Dis.* **50** (4), 231-235.

1034. Graham,D.Y. (1989) Campylobacter pylori and peptic ulcer disease. *Gastroenterology* **96** (2 Pt 2 Suppl), 615-625.

1035. Tytgat,G. and Dixon,M. (1993) Role in Peptic Ulcer Disease: Overview. In: Northfield,T.C., Mendall,M., and Goggin,P.M. (eds), pp. 75-87, Kluwer Academic Publishers., Dordrecht, The Netherlands.

1036. Dixon,M.F. (1994) Pathophysiology of Helicobacter pylori infection. *Scand.J.Gastroenterol.Suppl* **201** 7-10.

1037. Logan,R.P., Walker,M.M., Misiewicz,J.J., Gummett,P.A., Karim,Q.N., and Baron,J.H. (1995) Changes in the intragastric distribution of Helicobacter pylori during treatment with omeprazole. *Gut* **36** (1), 12-16.

1038. Elitsur,Y., Lawrence,Z., and Triest,W.E. (2002) Distribution of Helicobacter pylori organisms in the stomachs of children with H. pylori infection. *Hum.Pathol.* **33** (11), 1133-1135.

1039. Saitoh,K. and Ohkura,Y. (1998) [Histopathological study of the role of Helicobacter pylori in the occurrence and development of chronic active gastritis, acute erosive gastritis and gastric ulcer]. *Nippon Shokakibyo Gakkai Zasshi* **95** (1), 9-17.

1040. Baczako,K., Kuhl,P., and Malfertheiner,P. (1995) Lectin-binding properties of the antral and body surface mucosa in the human stomach--are the differences relevant for Helicobacter pylori affinity? *J.Pathol.* **176** (1), 77-86.

1041. Lueth,M., Sturegard,E., Sjunnesson,H., Wadstrom,T., and Schumacher,U. (2005) Lectin histochemistry of the gastric mucosa in normal and Helicobacter pylori infected guinea-pigs. *J.Mol.Histol.* **36** (1-2), 51-58.
1042. Terry,K., Williams,S.M., Connolly,L., and Ottemann,K.M. (2005) Chemotaxis plays multiple roles during Helicobacter pylori animal infection. *Infect.Immun.* **73** (2), 803-811.

1043. Yamaoka,Y., Kodama,T., Gutierrez,O., Kim,J.G., Kashima,K., and Graham,D.Y. (1999) Relationship between Helicobacter pylori iceA, cagA, and vacA status and clinical outcome: studies in four different countries. *J.Clin.Microbiol.* **37** (7), 2274-2279.

1044. Magnusson,P.K.E., Enroth,H., Eriksson,I., Held,M., Nyren,O., Engstrand,L., Hansson,L.E., and Gyllensten,U.B. (2001) Gastric cancer and human leukocyte antigen: distinct DQ and DR alleles are associated with development of gastric cancer and infection by Helicobacter pylori. *Cancer Res.* **61** (6), 2684-2689.

1045. Garcia,E., Carvalho,F., Amorim,A., and David,L. (1997) MUC6 gene polymorphism in healthy individuals and in gastric cancer patients from northern Portugal. *Cancer Epidemiol.Biomarkers Prev.* **6** (12), 1071-1074.

1046. Carvalho,F., Seruca,R., David,L., Amorim,A., Seixas,M., Bennett,E., Clausen,H., and Sobrinho-Simoes,M. (1997) MUC1 gene polymorphism and gastric cancer--an epidemiological study. *Glycoconj.J.* **14** (1), 107-111.

1047. Fox,J.G., Beck,P., Dangler,C.A., Whary,M.T., Wang,T.C., Shi,H.N., and Nagler-Anderson,C. (2000) Concurrent enteric helminth infection modulates inflammation and gastric immune responses and reduces helicobacter-induced gastric atrophy. *Nat.Med.* **6** (5), 536-542.

1048. Nomura,A.M., Hankin,J.H., Kolonel,L.N., Wilkens,L.R., Goodman,M.T., and Stemmermann,G.N. (2003) Case-control study of diet and other risk factors for gastric cancer in Hawaii (United States). *Cancer Causes Control* **14** (6), 547-558.

1049. Lee,S.A., Kang,D., Shim,K.N., Choe,J.W., Hong,W.S., and Choi,H. (2003) Effect of diet and Helicobacter pylori infection to the risk of early gastric cancer. *J.Epidemiol.* **13** (3), 162-168.

1050. Kono,S. and Hirohata,T. (1996) Nutrition and stomach cancer. *Cancer Causes Control* **7** (1), 41-55.

1051. Brown,L.M. (2000) Helicobacter pylori: epidemiology and routes of transmission. *Epidemiol.Rev.* **22** (2), 283-297.

1052. Kavermann,H., Burns,B.P., Angermuller,K., Odenbreit,S., Fischer,W., Melchers,K., and Haas,R. (2003) Identification and characterization of Helicobacter pylori genes essential for gastric colonization. *J.Exp.Med.* **197** (7), 813-822.

1053. Hennig,E.E., Mernaugh,R., Edl,J., Cao,P., and Cover,T.L. (2004) Heterogeneity among Helicobacter pylori strains in expression of the outer membrane protein BabA. *Infect Immun.* **72** (6), 3429-3435.

1054. Rasko,D.A., Wilson,T.J., Zopf,D., and Taylor,D.E. (2000) Lewis antigen expression and stability in Helicobacter pylori isolated from serial gastric biopsies. *J Infect Dis* **181** (3), 1089-1095.

1055. Syder,A.J., Guruge,J.L., Li,Q., Hu,Y., Oleksiewicz,C.M., Lorenz,R.G., Karam,S.M., Falk,P.G., and Gordon,J.I. (1999) Helicobacter pylori attaches to NeuAc alpha 2,3Gal beta 1,4 glycoconjugates produced in the stomach of transgenic mice lacking parietal cells. *Mol.Cell* **3** (3), 263-274.

1056. Sipponen,P. (1998) Natural course of Helicobacter pylori gastric infection. *Ital.J Gastroenterol.Hepatol.* **30 Suppl 3** S270-S272.

1057. Karita,M. and Blaser,M.J. (1998) Acid-tolerance response in Helicobacter pylori and differences between cagA+ and cagA- strains. *J.Infect.Dis.* **178** (1), 213-219.

1058. Rijpkema,S.G., Durrani,Z., Beavan,G., Gibson,J.R., Luck,J., Owen,R.J., and Auda,G.R. (2001) Analysis of host responses of guinea pigs during Helicobacter pylori infection. *FEMS Immunol.Med.Microbiol.* **30** (2), 151-156.

1059. Sturegard,E., Sjunnesson,H., Ho,B., Willen,R., Aleljung,P., Ng,H.C., and Wadstrom,T. (1998) Severe gastritis in guinea-pigs infected with Helicobacter pylori. *J.Med.Microbiol.* **47** (12), 1123-1129.

1060. Shomer,N.H., Dangler,C.A., Whary,M.T., and Fox,J.G. (1998) Experimental Helicobacter pylori infection induces antral gastritis and gastric mucosa-associated lymphoid tissue in guinea pigs. *Infect.Immun.* **66** (6), 2614-2618.

1061. De Bolos,C., Garrido,M., and Real,F.X. (1995) MUC6 apomucin shows a distinct normal tissue distribution that correlates with Lewis antigen expression in the human stomach. *Gastroenterology* **109** (3), 723-734.

1062. Yu,L.C., Yang,Y.H., Broadberry,R.E., Chen,Y.H., Chan,Y.S., and Lin,M. (1995) Correlation of a missense mutation in the human Secretor alpha 1,2-fucosyltransferase gene with the Lewis(a+b+) phenotype: a potential molecular basis for the weak Secretor allele (Sew). *Biochem.J* **312 (Pt 2)** 329-332.

1063. Koda,Y., Soejima,M., Liu,Y., and Kimura,H. (1996) Molecular basis for secretor type alpha(1,2)-fucosyltransferase gene deficiency in a Japanese population: a fusion gene generated by unequal crossover responsible for the enzyme deficiency. *Am.J Hum.Genet.* **59** (2), 343-350.

1064. Murata,K., Egami,H., Shibata,Y., Sakamoto,K., Misumi,A., and Ogawa,M. (1992) Expression of blood group-related antigens, ABH, Lewis(a), Lewis(b), Lewis(x), Lewis(y), CA19-9, and CSLEX1 in early cancer, intestinal metaplasia, and uninvolved mucosa of the stomach. *Am.J.Clin.Pathol.* **98** (1), 67-75.

1065. Nakagoe,T., Fukushima,K., Sawai,T., Tsuji,T., Jibiki,M., Nanashima,A., Tanaka,K., Yamaguchi,H., Yasutake,T., Ayabe,H., and Ishikawa,H. (2002) Increased expression of sialyl Lewis(x) antigen in penetrating growth type A early gastric cancer. *J Exp.Clin.Cancer Res.* **21** (3), 363-369.

1066. Nakamori,S., Arai,I., Okamura,S., Imaoka,S., Furukawa,H., Kabuto,T., Ishikawa,O., Sasaki,Y., Kameyama,M., and Iwanaga,T. (1996) [Clinical value of carbohydrate antigens, sialyl Lewis-x and sialyl Lewis-a in gastrointestinal cancer]. *Nippon Geka Gakkai Zasshi* **97** (2), 165-171.

1067. Sumikura,S., Ishigami,S., Natsugoe,S., Miyazono,F., Tokuda,K., Nakajo,A., Okumura,H., Matsumoto,M., Hokita,S., and Aikou,T. (2003) Disseminated cancer cells in the blood and expression of sialylated antigen in gastric cancer. *Cancer Lett.* **200** (1), 77-83.

1068. Ugorski,M. and Laskowska,A. (2002) Sialyl Lewis(a): a tumor-associated carbohydrate antigen involved in adhesion and metastatic potential of cancer cells. *Acta Biochim.Pol.* **49** (2), 303-311.

1069. Kim,M.J., Kim,H.S., Song,K.S., Noh,S.H., Kim,H.G., Paik,Y.K., and Kim,H.O. (2002) Altered expression of Lewis antigen on tissue and erythrocytes in gastric cancer patients. *Yonsei Med.J.* **43** (4), 427-434.

1070. Teixeira,A., David,L., Reis,C.A., Costa,J., and Sobrinho-Simoes,M. (2002) Expression of mucins (MUC1, MUC2, MUC5AC, and MUC6) and type 1 Lewis antigens in cases with and without Helicobacter pylori colonization in metaplastic glands of the human stomach. *J Pathol.* **197** (1), 37-43.

1071. Lopez-Ferrer,A., De Bolos,C., Barranco,C., Garrido,M., Isern,J., Carlstedt,I., Reis,C.A., Torrado,J., and Real,F.X. (2000) Role of fucosyltransferases in the association between apomucin and Lewis antigen expression in normal and malignant gastric epithelium. *Gut* **47** (3), 349-356.

1072. Imura,H., Takahashi,T., Matsuda,T., Yoshida,O., Ohkura,H., Seitetsu,Y., Seino,Y., Ishii,M., Kuwabara,M., Ariyoshi,Y., and . (1989) [Basic and clinical evaluation of an immunoradiometric competitive inhibition assay for 2----6 sialyl Lewis a antigen--2. Evaluation of clinical significance]. *Gan To Kagaku Ryoho* **16** (6), 2203-2212.

1073. Itai,S., Nishikata,J., Yoneda,T., Ohmori,K., Yamabe,H., Arii,S., Tobe,T., and Kannagi,R. (1991) Tissue distribution of 2-3 and 2-6 sialyl Lewis A antigens and significance of the ratio of two antigens for the differential diagnosis of malignant and benign disorders of the digestive tract. *Cancer* **67** (6), 1576-1587.

1074. Henry,S.M., Benny,A.G., and Woodfield,D.G. (1990) Investigation of Lewis phenotypes in Polynesians: evidence of a weak secretor phenotype. *Vox Sang.* **58** (1), 61-66.

1075. Linden,S., Mahdavi,J., Hedenbro,J., Boren,T., and Carlstedt,I. (2004) Effects of pH on Helicobacter pylori binding to human gastric mucins: identification of binding to non-MUC5AC mucins. *Biochem.J.* **384** (Pt 2), 263-270.

1076. Prinz,C., Schoniger,M., Rad,R., Becker,I., Keiditsch,E., Wagenpfeil,S., Classen,M., Rosch,T., Schepp,W., and Gerhard,M. (2001) Key importance of the Helicobacter pylori adherence factor blood group antigen binding adhesin during chronic gastric inflammation. *Cancer Res.* **61** (5), 1903-1909.

1077. Yamaoka,Y., Kwon,D.H., and Graham,D.Y. (2000) A M(r) 34,000 proinflammatory outer membrane protein (oipA) of Helicobacter pylori. *Proc.Natl.Acad.Sci.U.S.A* **97** (13), 7533-7538.

1078. Rokbi,B., Seguin,D., Guy,B., Mazarin,V., Vidor,E., Mion,F., Cadoz,M., and Quentin-Millet,M.J. (2001) Assessment of Helicobacter pylori gene expression within mouse and human gastric mucosae by real-time reverse transcriptase PCR. *Infect Immun.* **69** (8), 4759-4766.

1079. Gurbuz,A.K., Ozel,A.M., Ozturk,R., Yildirim,S., Yazgan,Y., and Demirturk,L. (2005) Effect of N-acetyl cysteine on Helicobacter pylori. *South.Med.J* **98** (11), 1095-1097.

1080. Yuta,A. and Baraniuk,J.N. (2005) Therapeutic approaches to mucus hypersecretion. *Curr.Allergy Asthma Rep.* **5** (3), 243-251.

1081. Langermann,S., Palaszynski,S., Barnhart,M., Auguste,G., Pinkner,J.S., Burlein,J., Barren,P., Koenig,S., Leath,S., Jones,C.H., and Hultgren,S.J. (1997) Prevention of mucosal Escherichia coli infection by FimH-adhesin-based systemic vaccination. *Science* **276** (5312), 607-611.

1082. Anantha,R.P., McVeigh,A.L., Lee,L.H., Agnew,M.K., Cassels,F.J., Scott,D.A., Whittam,T.S., and Savarino,S.J. (2004) Evolutionary and functional relationships of colonization factor antigen i and other class 5 adhesive fimbriae of enterotoxigenic Escherichia coli. *Infect Immun.* **72** (12), 7190-7201.

1083. Katz,D.E., DeLorimier,A.J., Wolf,M.K., Hall,E.R., Cassels,F.J., van Hamont,J.E., Newcomer,R.L., Davachi,M.A., Taylor,D.N., and McQueen,C.E. (2003) Oral immunization of adult volunteers with microencapsulated enterotoxigenic Escherichia coli (ETEC) CS6 antigen. *Vaccine* **21** (5-6), 341-346.

1084. Singleton,D.R. and Hazen,K.C. (2004) Differential surface localization and temperature-dependent expression of the Candida albicans CSH1 protein. *Microbiology* **150** (Pt 2), 285-292.

1085. Trinel,P.A., Jouault,T., Cutler,J.E., and Poulain,D. (2002) Beta-1,2-mannosylation of Candida albicans mannoproteins and glycolipids differs with growth temperature and serotype. *Infect Immun.* **70** (9), 5274-5278.